The hen's eggshell: A resistance network.

Sparks, N. H. C.

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THE HEN'S EGGSHELL - A RESISTANCE NETWORK

Submitted by N.H.C. Sparks

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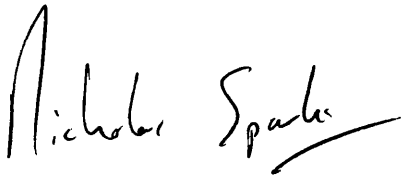

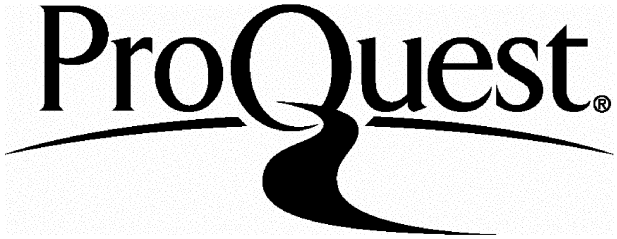

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SUMMARY

The formation and the fine structure of the components (cuticle, porous calcitic shell, shell membranes and limiting membrane) of the integument of the hen's egg were studied in detail with Scanning Electron Microscopy or, when appropriate, Transmission Electron Microscopy, in order to build up a detailed picture of the physical antimicrobial systems of the egg pre- and post-oviposition. This phase of the study revealed that (i) the limiting membrane is present on the egg in the magnum, (ii) the interstitial spaces of the shell membranes in the oviduct are filled with amorphous material which probably condenses to form the mantle on the individual fibres of the membranes and (iii) the cuticle at oviposition is immature and mechanically weak.

The immature cuticle is an ineffective barrier to bacterial penetration for a few minutes post-oviposition. When the components of the integument were considered as resistances in series, the mature cuticle - especially that on brown eggs - was shown to be the major barrier to the movement of water in the liquid but not the vapour state. Consequently this structure plays an important role in protecting the egg contents from infection. Work needs to be done in order to overcome this resistance - differences in partial pressure caused by immersing warm eggs in a cold solution overcame the cuticle's resistance. The efficacy of this method was enhanced by reducing the surface tension or "degassing" the water. Of the many methods used to assess cuticle quality, staining was the only one that would appear to be of any use at the moment in a breeding programme that sought improvements in the quality and hence effectiveness of the cuticle as a barrier to infection.

The porosity of the shell, as measured by G_{H_2O}, was not associated with water uptake by eggs. Nevertheless the pore contributed to the waterproofing, and hence antimicrobial defence of the egg, by imposing such a resistance to water movement that the osmotic potential of the albumen to pull water across the shell membranes was negated.

The shell membranes proved to be ineffective barriers to microbial movement. The contribution of the limiting membrane to the egg's defence could not be assessed with the techniques used in this study.

The environment within the shell membranes was selective for Gram-negative bacteria. Indeed the membranes appeared to offer a haven for microorganisms that had passed across the shell, especially in infected eggs held under conditions recommended for hatching eggs before transfer to incubation temperature. In practice viable organisms persisted in these structures whereas contaminants of the underlying albumen died out especially with incubation temperatures approaching the body temperature of the hen. Heavy microbial challenges ($>10^6$/egg) to the shell membranes resulted in heavy contamination of the albumen within two days of infection. It was deduced that contaminants of the albumen failed to multiply unless they made contact with the yolk.

The above observations were discussed in the context of practices that should be considered by hatchery managers who seek to interrupt the transfer of Salmonella from the breeding to the rearing flock by egg treatment. The identification of the immature cuticle state directs attention to the management and hygiene of nest boxes and the primary role of the cuticle in the eggs antimicrobial defence. It suggests that breeding programmes ought to be concerned with the quality of the cuticle throughout the laying cycle. The influence

of temperature on the behaviour of contaminants in the shell membranes suggest also that treatment of eggs with germicides to control *Salmonella* transmission needs to be done.

INTRODUCTION

INTRODUCTION

The studies by Savage and White (1925) led to Salmonella being recognized as a cause of food poisoning in Britain. Until the Second World War, however, it was generally assumed that salmonellosis was associated with human carriers infecting food during preparation. When laboratories started to isolate previously unknown ("exotic") serotypes of Salmonella from dried/frozen egg imported into the UK from the USA and from infected humans, a link was established between a commodity and a disease without evidence of direct human involvement. Indeed the realization that Salmonella contamination occurred during preparation from sources, principally the eggshell, other than humans led to legislation - The Liquid Egg (Pasteurisation) Regulations 1963 - prohibiting the use of unpasteurised liquid egg (yolk or albumen) as an ingredient in the preparation of food. As a consequence of these regulations processed egg products have ceased to be a major cause of salmonellosis in the human population of the UK.

Recently it has been appreciated that the eggs of hens and turkeys still provide an important route for the dissemination of salmonellae from breeding sites through commercial hatcheries to birds being grown for slaughter (Stuart, 1984). In many instances the salmonellae are present in the feed of breeding flocks (Williams, 1981a, b). The salmonellae become established in the intestines and those passed in faeces can contaminate the shell and subsequently the interior of hatching eggs. It is accepted by some that one way to gain freedom from salmonella contamination of the processed bird (the largest single source of salmonellas in Britain (Roberts, 1982)) would be to achieve a salmonella-free state in

the bird at the time of slaughter. Others contend that racidation (treatment with ionizing radiation at doses up to 5 kGy) of the processed bird would provide an adequate safeguard to the consumer (Kampelmacher, 1983). Apart from attempts to control contamination of feeds, the most common means for the introduction of salmonellae into a poultry flock (Williams, 1981a, b), certain other practices are being investigated with the first objective in mind *viz* decontamination of the shell of hatching eggs and protection of the newly hatched chick (Pivnick and Nurmi, 1982).

Ideally the eggs selected for hatching are nest clean; "floor eggs" or those with dirty or damaged shells being rejected because of the increased risk of bacterial penetration (Smeltzer *et al.*, 1979). In practice, of course, hatchery managers' concern with salmonellae is often subordinate to that of rot-producing organisms. The shells of eggs selected for hatching are sanatized (normally by fumigation with formaldehyde) before setting (Furuta and Sato, 1977a). Indeed after this point it is very difficult to remedy faults in management because, on hatching, contaminated embryos and those breaking out through contaminated shells will cause *Salmonella* to be spread throughout the hatchery thereby putting all chicks at risk.

A novel treatment aimed at the newly hatched chick was introduced and named after Nurmi (Pivnick and Nurmi, 1982) as a supplement to egg treatments (Nurmi and Rantala, 1973). The Nurmi concept is based on competitive exclusion and involves *per os* introduction of gastrointestinal flora from adult birds into newly hatched chicks or poults, thereby endowing them with resistance to 10^5 *Salmonella* cells per chick.

At the time when poorly controlled egg washing and

subsequent long term storage of shell eggs led on many occasions to high incidences of rotting (Moats, 1978), it was assumed that a rot was caused by bacteria that had penetrated the shell after oviposition (Lorenz et al., 1952). Indeed the available evidence suggests that the vast majority of hens' eggs are germ free at oviposition (Board, 1966). Bacterial penetration of the shells of hen and turkey eggs has again attracted attention because in the opinion of some (Marthedal, 1973; Stuart, 1984) this may be an essential step in the transfer of salmonellas from breeding stock to birds reared as broilers and eventually to the kitchen in the home and canteen. Indeed much effort is being given to methods that free hatching eggs, for example, from food poisoning bacteria. Some investigators are attempting to introduce solutions of germicides into the pore canals of eggs so that bacteria that have infected the shell membranes are killed without impairment of embryo development (Alls et al., 1963; Furuta and Sato, 1977b). It needs to be stressed, however, that this work is being hindered because of the fragmentary nature of much of our knowledge about the fine structure of the egg integument - the calcitic shell clothed on its outer surface with cuticle and on its inner one with two shell membranes and the poorly defined limiting membrane - and factors that contribute to its penetration by bacteria. In some recent publications (Tullett and Board, 1976; Board, 1980; Board, 1982) this integument has been discussed in terms of resistances in series (Fig. 1). The objectives of the present study were: (i) to define in greater detail the fine structure of the components of the integument, especially the limiting membrane occurring on the inner surface of the shell membrane; (ii) to assess the efficacy

of the components of the integument as barriers to bacterial invasion of the shell; and (iii) to study the fate of a mixed bacterial infection on the shell membrane of eggs incubated at 37.5°C.

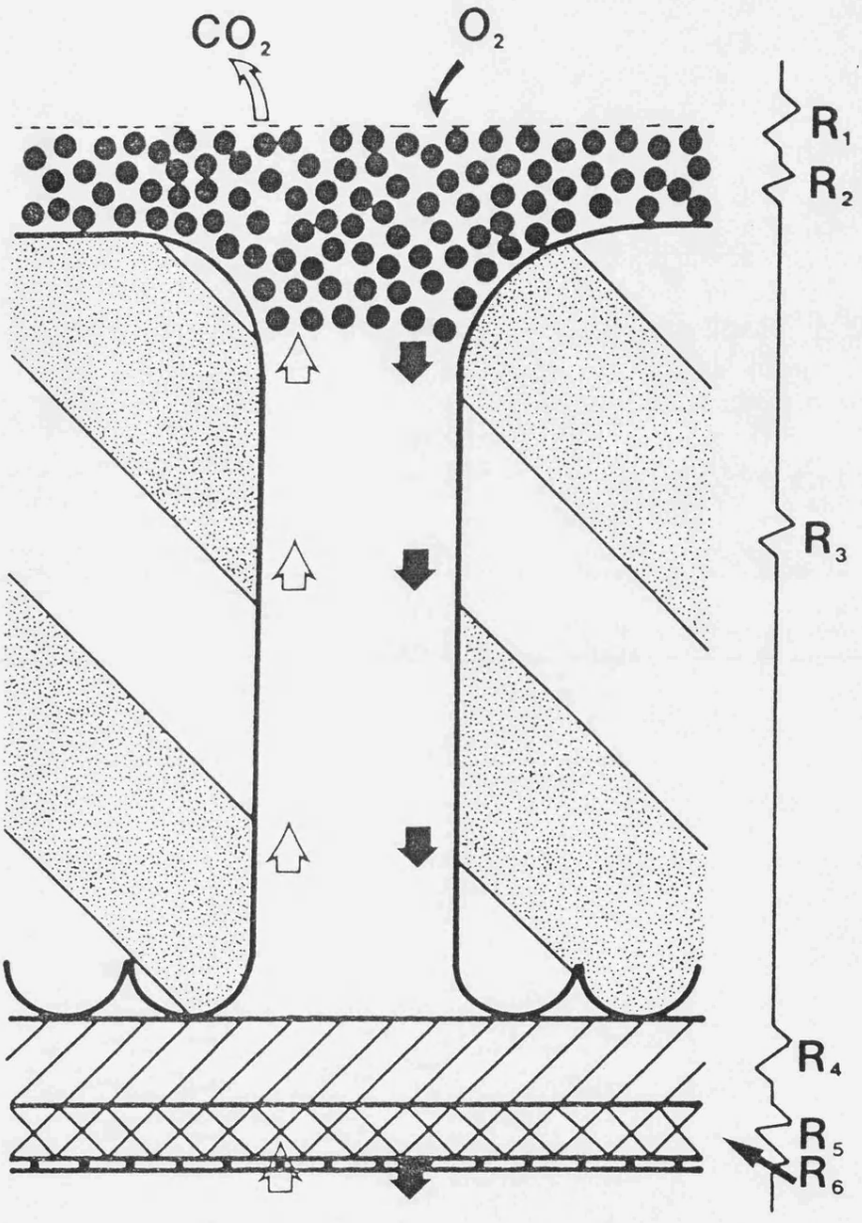


Fig. 1 The egg integument considered as a series of resistances in series: R_1 - boundary layer, R_2 - cuticle, R_3 - pore canal, R_4 - outer shell membrane, R_5 - inner shell membrane, R_6 - limiting membrane.

(from Tranter et al., 1983).

LITERATURE REVIEW

THE RESISTANCE NETWORK - STRUCTURE

LITERATURE REVIEW

The resistance network (Fig. 1) of the egg integument comprises the cuticle, shell (pore system), shell membrane and limiting membrane. When assessing an egg's ability to resist penetration by liquids and foreign bodies it is essential to identify the effectiveness of each resistance. For instance when Kraft et al. (1958) attempted to determine the factors that influence bacterial penetration of eggshells, they considered the integument as a whole and consequently overlooked the crucial role of the cuticle (Board and Halls, 1973a). Before a study of egg shell penetration is attempted there is a need therefore to understand the fine structure of the resistances involved otherwise it is difficult to consider their probable mode of operation (ie shell membranes acting as bacterial filters (Haines and Moran, 1940)). The literature on the resistance network has been reviewed therefore and the components (Fig. 1) studied in the context of fine structure and their effectiveness in preventing bacterial and fluid penetration of the egg.

THE RESISTANCE NETWORK

THE CUTICLE AND COVER

The outer surface of the calcitic shell of domestic hens, ducks and turkeys is generally covered by an organic cuticle (Cooke and Balch, 1970) which is probably secreted in the shell gland as a granular substance (McCallion, 1953; Cooke and Balch, 1970). The nature and distribution of the secretory cells have yet to

be identified.

At oviposition the cuticle is a slippery lustrous layer which dries rapidly on exposure to the atmosphere. The reddish-brown pigmentation of some cuticles is associated with the occurence of ooporphyrin - protoporphyrin - (Fischer and Kögl, 1923, 1924; Fischer and Müller, 1925), a derivative of blood haemoglobin (Romanoff and Romanoff, 1949). Accumulations of porphyrin grains result in brown spots (Schmidt, 1958). Ooporphyrin is also present in white eggshells (Völker, 1940) but at such low concentrations that ultra-violet light must be used for its detection (red fluorescence).

Some hens' eggs are oviposited in a cuticle-less state. Board and Halls (1973a) found 3.5% (453) of brown eggs to be cuticle-less whilst 8.0% were devoid of cuticle at one or other of the poles. Reasons for the total or partial cuticle-less state are unknown (Board, 1975).

In electron microscopy studies the outer surface of the cuticle appears to be uneven with many star-shaped cracks, fissures and flake-like layers evident (Parsons, 1982). The larger cracks, having a roughly radial orientation, commonly occur over the outer pore orifice (Simons, 1971). In radial section the cuticle is seen to be unevenly distributed, its thickness varying from 0.5 - 12.8 μm at different places on the shell of the same egg (Simons, 1971).

Baudrimont and St Ange (1847) considered the cuticle to be a structureless epithelial layer. Other studies (Krampitz and Witt, 1979) have indicated that the cuticle is perhaps a composite structure. Indeed an outer granular layer and an inner structureless one was described by Romankewitsch (1934)

and an outer homogeneous membrane (sic) 50 mμm thick and an organic layer 0.5 - 2.5 μm thick by Skalinsky (1966). Simons (1971) found the cuticle to have a vesicular structure in decalcified sections of hens' eggshell, the spherical and ovoid vesicles, which were up to 1 μm in diameter, were empty or partially filled with granular material. He also reported that the outer quarter, which contained many vesicles of 0.2 μm diam., was more compact than the rest of the cuticle. This might account for Skalinsky's (1966) observations.

The cuticle consists of ca. 90% protein (Baker and Balch, 1962); the amino acid pattern differs from that of the shell membranes in having a high glycine content (Krampitz and Witt, 1979). The carbohydrate moiety of the proteoglycan in the cuticle consists of sialic acid, galactosamine, galactose, glucose, mannose and fucose; uronic acids and acidic mucopolysaccharides have not been detected (Balch and Cooke, 1970).

PORES

Pores, which were first investigated systematically by von Nathusius (1821 - 1899, see Tyler, 1964), traverse radially the shell of all avian eggs (Plate 1). The post horn shaped pores (ca. 7,000 - 17,000 per egg, Simkiss, 1968) of the domestic hen egg are capped over their outer orifice (15 - 65 μm diam.) with organic material (Board et al., 1977), the inner orifice (6 - 23 μm diam.) being unobstructed (Simkiss, 1968). The density of pores appears to be greatest at the equator or blunt pole of the eggshell (Romanoff and Romanoff, 1949), and their distribution is not random but tends towards uniformity (Tyler, 1969a).

Von Nathusius (1893 - see Tyler, 1964) believed that the pores arose from matrix threads extending radially from the outer shell membrane, a hypothesis in accord with that of Stewart (1935) who surmised that protein material within the crystalising mass formed threads which, after shell formation, dried away from the pore sides thereby allowing gaseous diffusion, the primary physiological role of the pores (Hoyt et al., 1979). Whilst studying eggshell formation, Schmidt (1966) observed that an incomplete fusion of the cone tips resulted in spaces remaining between them. In studies with an electron microscope, Tullett (1975) also came to this conclusion, finding a positive linear correlation between the number of cone tips/cm^2 and the number of pores/cm^2 for domestic hen eggshells and those of 33 other avian species. Tullett suggested that the relationship reflected the irregular packing of the calite columns, which was controlled by the density of the seeding sites on the outer shell membrane. Indeed Wyburn et al. (1973) believed that the location of these seeding sites - and hence the porosity of eggshells - is determined by the distribution of the uterine cells which secrete the nuclei (cores) of crystalisation. Tullett (1975) opined that, once initiated, the presence of uterine fluid at the crystal interface, as suggested by Tyler and Simkiss (1959), keeps a pore open during shell formation. It is noteworthy that Tyler (1969b) found the pigmented layer on the surface of Phaethontidae shells lined the pore canals for up to 25% of the shell thickness; this suggests a dynamic state within the pore lumen at the time of deposition rather than the static one suggested by the protein thread hypothesis.

Tullett (1975), who found that the inner orifice of all pore canals were bounded by not less than four cones, put emphasis

on the importance of cone location in pore initiation. Recently Tyler and Fowler (1978) and Silyn-Roberts (1983) have reported that the pore origin may be surrounded by as few as three cones. Tyler and Fowler (1978), who used a different method for pore counting to that of Tullett (1975), could find no correlation between pore/cone numbers, pore/core numbers or pore/cone junctions. It needs to be noted that the methods of Tyler and Fowler (1978), based as they were upon studies with the light microscope, may not have provided adequate information. Although core numbers were not considered in this study the pore canals of duck and turkey eggs were examined for evidence of proteinacous material (see pp 31).

Whatever the mechanism of pore initiation many fail to develop fully, resulting in blind canals. These are readily identified with plastic moulds of the cone layer and pore canals (Fujii, 1974).

Board and Halls (1973a) used plastic pore models specifically to study pore configuration and its effect on particle penetration. They found, as did Fujii (1974), that some shells contained a few malformed - larger than average - pores. Such pores, often called "patent pores" in contemporary literature, were discussed by Conrad and Scott (1938) who believed that their unusual size was perhaps associated with a malfunction of the mechanism that led to the threads that Stewart (1935) considered to be important in pore formation. Such pores have been identified as major portals for the ingress of bacteria (Board and Halls, 1973a) and antibiotics (Alls *et al.*, 1964).

SHELL MEMBRANES

The avian eggshell membranes are characterised by two distinct layers, the inner and outer membranes, each of which is made up of layers of coaxial fibres randomly orientated in the tangential plane (Bellairs and Boyde, 1969). The spaces between the fibres of the membranes of fully formed eggs are filled with air (Simons, 1971). The domestic hens' shell membranes, which are on average 48 μm (outer) and 22 μm (inner) thick, are loosely fused together by the mantles on the fibres except at the broad pole where they commonly separate to form the so called air space (Simons, 1971). In a detailed study of the dimensions of the membranes Balch and Tyler (1964) found that not only was shell membrane thickness not uniform (range of 93 - 115% of the mean thickness) around the egg, but that the variation was greatest from pole-to-pole and minimal around a given latitude.

When the membranes are viewed in radial section the fibres of the inner appear to be smaller in diameter and more densely packed than those of the outer membrane (Simons, 1971). The diameter of the fibres in the membranes of domestic hens varies between 0.4 - 3.6 μm, the inner membrane fibres seldom exceed 2.0 μm (Bellairs and Boyde, 1969).

When viewed with the transmission electron microscope (TEM) it is evident that the fibres consist of a homogeneous electron dense core and a less dense, occasionally granulated, outer layer or mantles (Masshoff and Stolpmann, 1961). The core represents the fibre proper while the mantle appears to act as a coating which cements them into a network (Hoffer, 1971). In the membranes of the chicken eggshell the core diameter averages 0.8 - 1.0 μm, with a mantle thickness of 0.5 μm. The core and mantle appear to be separated by a space of 0.1 - 0.3 μm in which fibrils of 0.0016 μm

diam. occur (Masshoff and Stolpmann, 1961). The fibres tend to uniformity along their length although both inner and outer fibres may possess isolated or aggregated small (0.5 - 1.5 μm diam.) round/oval protuberances (Bellairs and Boyde, 1969).

The membranes are almost entirely proteinaceous, less than 4% (dry weight) being carbohydrate and of this 70 - 80% are neutral sugars consisting of a mixture of galactose, glucose, mannose and fucose. Small amounts of sialic acid, glucosamine and galactosamine are present (Balch and Cooke, 1970). Wedral et al. (1974) found the membrane contained on average 2.8% lipids.

Early studies by Calvery (1933), Wolken (1951) and Baker and Balch (1962) suggested that, because of its high content of SH groups, the major protein was similar to keratin. This led to the term ovokeratin being coined. Recent studies however (Baumgartner et al., 1978; Harris et al., 1980) have demonstrated the presence of desmosine and isodesmosine in hydrolysates of the membranes, amino acids that were once considered to be uniquely characteristic of the connective tissue protein, elastin. That the membrane protein is not elastin was shown by several studies (Starcher and King, 1980; Crombie et al., 1981; Leach et al., 1981), as not only did it "differ greatly" from elastin in its amino acid composition but it was also resistant to digestion with elastase. This major "elastin-like" protein component, characterised by its disulphide crosslinks and its lysine derived crosslinks of desmosine and isodesmosine (Fig. 2), accounts for 70 - 75% of the membrane protein; collagen accounts for another 10%, the remainder being a glycoprotein (Leach, 1982). Although the mantle is classed as a glycoprotein (Balch and Cooke, 1970), there is no evidence to support the suggestion of Masshoff and

Stolpmann (1961) that the carbohydrate is confined exclusively to this structure.

The fibrous shell membranes of the domestic hen eggshell are formed in the upper two thirds or granular region of the isthmus. The inner surface of the isthmus consists of numerous longitudinal ridges, the surface epithelium of which is comprised of columnar cells below which is a layer of tubular glands (Draper et al., 1972). It is the prominent secretory granules within the tubular gland cells that are responsible for the name granular isthmus. Although tubular cells are also present in the lower third of the isthmus they contain fewer secretory granules (Draper et al., 1972).

Draper et al. (1972) identified the tubular gland as being responsible for the synthesis of membrane protein. The luminal surface of this gland is composed of cells which Hoffer (1971) designated "principal" cells. The protein is synthesised on ribosomes associated with an extensive endoplasmic reticulum (ER). The products of this association are concentrated in the lumen of the ER and transported to the Golgi region which forms secretory droplets. The tubular glands then excrete the thread-like fibres, coated with a mantle, into the lumen of the oviduct where they are deposited on the rotating egg mass. Hoffer (1971) found the Golgi apparatus to be prominent in principal cells and postulated that it was also the main site for synthesis of the carbohydrate moiety of glycoprotein secretory products. The preponderance of granular material in the surface epithelium cells led Draper et al. (1972) to propose that they contributed a protein fraction to the secretions of the granular isthmus; they conceded, however, that more precise cyto-chemical techniques were needed to

define the specific nature of the secretions of mucosal cells.

LIMITING MEMBRANE

The limiting membrane, a smooth, electron dense (0.25 - 0.75 µm thick, Froix et al., 1977), homogeneous sheet (Candlish, 1972) is interposed between the albumen and the inner shell membrane (Fig. 1). Although noted by von Nathusius in 1893 (Tyler, 1964) and found to be deposited around the albumen at the aglandular junction of the magnum/isthmus (Draper et al., 1972), it has been almost totally ignored in most subsequent studies or dismissed as an artefact of preparative techniques. The term "limiting membrane", as used by Bellairs and Boyde (1969), not only served to rekindle interest in the structure found at the interface of the albumen and shell membranes but offered an alternative to vague descriptions such as "a narrow 50 mµm thick fringed layer of the shell membrane" (Skalinsky, 1966) or the "inner layer of the inner membrane" (Simons and Wiertz, 1963). It has been noted primarily in domestic hen eggs "and as such might be peculiar to the Galliformes" (Becking, 1975).

The chemical composition and origin of the limiting membrane are uncertain. Bellairs and Boyde (1969) were of the opinion that its composition was identical to that of material in the mantle on the membrane fibres. They surmised that this material had properties which caused it to "spread and cover any surface with which it comes into contact", thereby giving rise to the limiting membrane as well as coating the fibre cores. Simons (1971) also expressed the view that the limiting membrane was composed, in part, of the same granular material that comprised the mantle

of the fibres. The shell membranes of the Japanese quail were studied by Hoffer (1971) who found material similar in appearance to the limiting membrane in the interstices of the shell membranes. Hoffer was of the opinion that this represented either a less condensed form of the mantle materials, thereby supporting the contention of Bellairs and Boyde (1969) and Simons (1971), or evidence for a matrix that had been largely removed during specimen preparation.

Thus it is evident from the paucity of information that there is a need for further studies of the limiting membrane and, in the context of the egg integument as a resistance, a method for determining whether or not it constitutes a barrier to the movement of materials into the egg. This topic is discussed on pp 265.

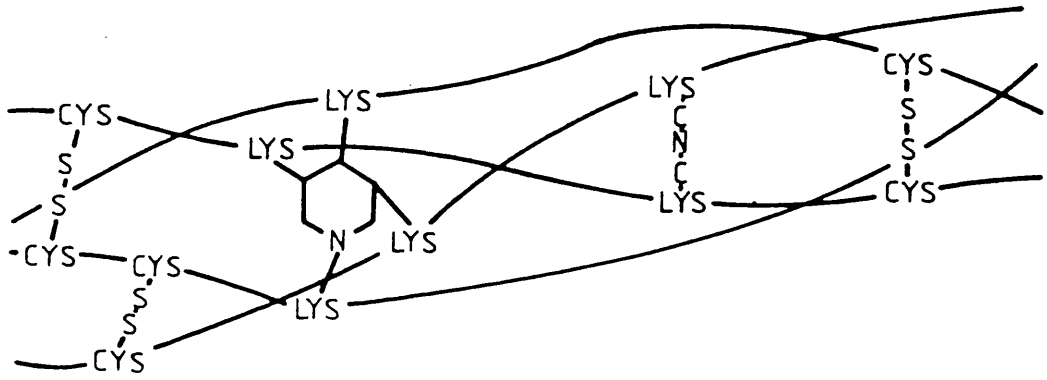
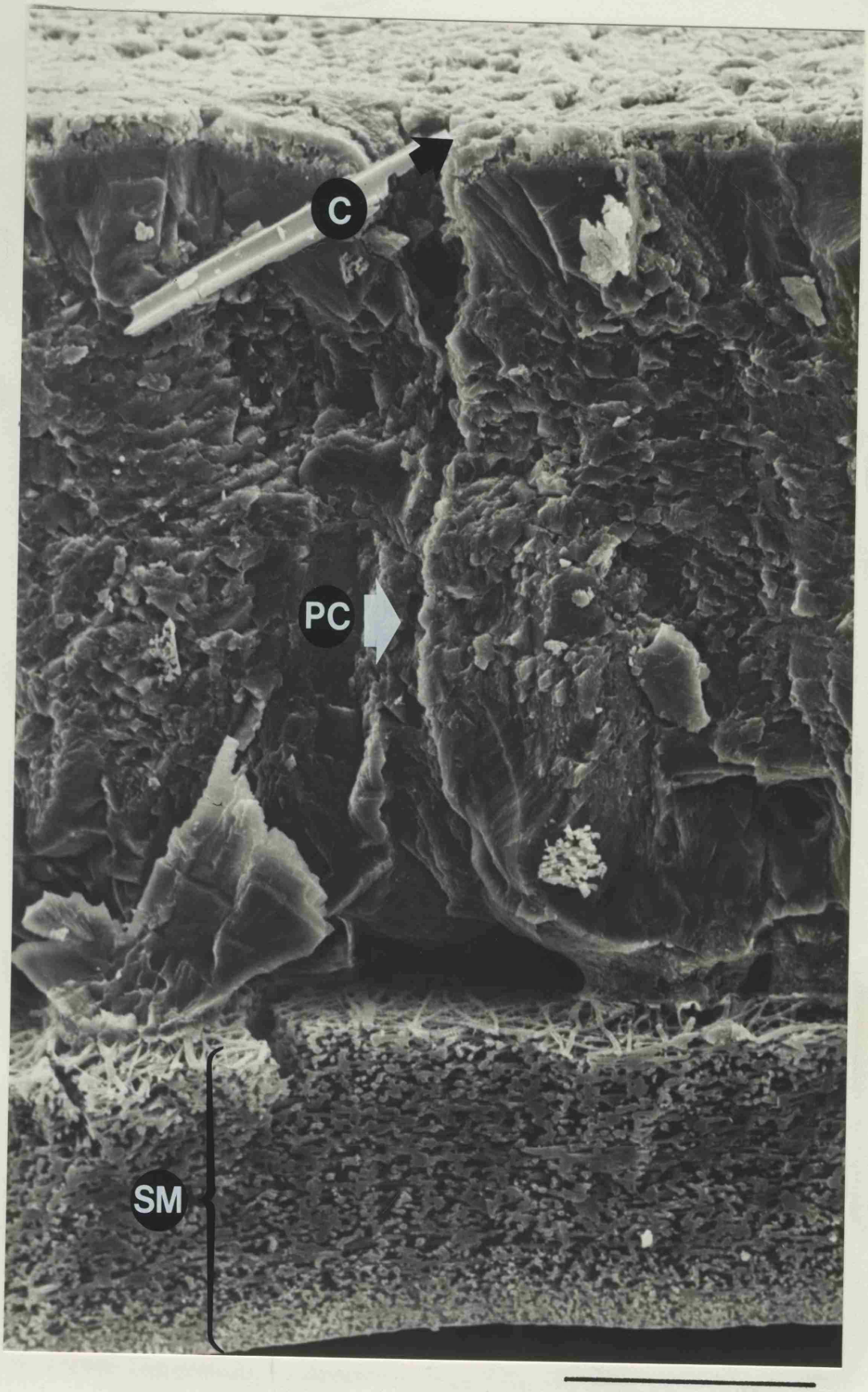


Fig. 2 Hypothesised structure of eggshell membrane protein. The peptide chains are cross-linked by both disulphide and lysine derived cross-links (from Leach, 1982).

PLATE 1. Radially fractured Pink-eared duck eggshell showing the pore canal (PC), shell membranes (SM) and cuticle (C).
Bar marker 100μm.


C
PC
SM

MATERIALS AND METHODS

THE RESISTANCE NETWORK - STRUCTURE

MATERIALS AND METHODS

INTRODUCTION

Electron microscopy

Scanning (SEM) and transmission (TEM) electron microscopy samples were prepared as follows:

SEM (freeze dried)

Large sections of shell were frozen in liquid N_2 and fractured, the resultant pieces being freeze dried (Edwards, Crawley, Sussex). The samples were then mounted with graphite paste (Polaron Equipment Ltd., Watford, Herts.) onto aluminium planchettes, sputter-coated in vacuo with a thin layer of gold/paladium (Polaron Equipment Ltd., Watford, Herts.) and examined using a J35C SEM (Jeol (UK) Ltd., Colindale, London).

SEM (critical point dried)

Samples were fixed in 0.2M gluteraldehyde in cacodylate buffer (pH 7) for 18h at room temperature followed by a series dehydration in 30-100% acetone. The samples were dried to critical point using a critical point dryer (Polaron Equipment Ltd., Watford, Herts.) and mounted on aluminium planchettes, coated and viewed as described above.

TEM

Samples were fixed in 0.2M gluteraldehyde (in a cacodylate buffer) containing 0.1% (w/v) ruthenium red, washed with cacodylate, fixed in 1% osmium tetroxide (1h), washed again in cacodylate and then series dehydrated as described above. The samples were embedded in Taab EM resin (Taab Laboratories Equipment Ltd., Reading, Berks.). Ultra-thin sections were cut

with a glass knife and collected onto copper grids, staining being carried out as described by Forsdyke (1981) with a saturated solution of uranyl acetate in ethanol (70 ml/l) and lead citrate (Reynolds, 1963). The sections were examined using a J100CX transmission electron microscope (Jeol (UK) Ltd., Colindale, London).

Source of eggs

See Table 1.

CUTICLE

Cuticle staining

The cuticle was stained with Edicol Supra Pea Green H (ICI, Hexagon House, Blackley, Manchester) as described by Board and Halls (1973a). Edicol Supra Pea Green H's two moieties, Tartrazine - 1409 and Green S - 12947 (D.F. Anstead Ltd., Billericay, Essex), were also used individually to stain the cuticle. The concentrations of Green S and Tartrazine (2.8g/l and 7.2g/l respectively) were identical to those in Edicol Supra Pea Green H.

Cuticle staining - individually caged birds

Six pullets and six hens (Ross Ranger) were individually caged in traditional roll-away battery cages. The eggs were collected in sequence and stained as described above.

Cuticle removal and weight

The eggs were immersed for 15 minutes in a 10% (w/v)

alkaline (pH 7.5) solution of ethylenediaminetetraacetic acid (EDTA). The cuticle was then removed with a fine jet of water.

The cuticle weight was determined by removing the cuticle with EDTA, harvesting it on a preweighed glass microfibre filter (Whatman, Maidstone, Kent) and, after adequate washing, drying to a constant weight.

Cuticle crack length

Samples of shell were freeze dried and micrographs of the cuticle taken using the J35C SEM at a magnification of x130. A transparent film with a ruled grid (9 x 6 squares) was laid over the micrograph and the cuticle cracks traced for the required square (determined by the use of random numbers), on to tracing paper - 10 squares in total for each egg (five squares on each of two micrographs). Crack length was measured with a map measure (Polco Products Ltd., Brentford, Middx.).

Fine structure of the "wet" and "dry" cuticle

Six hens (Light Sussex x Rhode Island Red) were individually housed in roll-away battery cages. The birds were observed and immediately an egg was oviposited it was removed - taking care to handle only the sharp/blunt ends - and placed in 0.2M gluteraldehyde in 0.5M cacodylate buffer (pH 7). After 18h the egg was removed, washed five times with deionised water and a $1cm^2$ section cut from the shell. The samples were then critical point dried as discussed above and viewed on the SEM. The next egg in the clutch was used as a control, the cuticle being allowed to dry (room temp.) over a period of 1h before fixing.

LIMITING AND SHELL MEMBRANES

Specimen preparation

Pieces of shell integument approximately $1cm^2$ were removed using a high speed diamond-tipped cutting wheel (Cahill Dental Ltd., Henleaze, Bristol), adhering albumen was washed free with deionised water and the sample was prepared as described above - SEM (freeze dried) and TEM.

DEVELOPMENT OF THE EGG IN UTERO

Source

Eggs were obtained from the oviduct of freshly (<10 mins) slaughtered Aylesbury ducks and turkeys.

Preparation

Pre-oviposited eggs were treated in one of three ways - SEM (freeze dried) as described above, TEM also described above or cryogenic SEM.

Cryogenic SEM involves plunging the sections (mounted on the specimen holder with graphite) into slush nitrogen (ca. $-210^{\circ}C$) and then moving the specimen from the slushing chamber to the pre-chamber via a transfer device which maintains a constant temperature and prevents contamination with atmospheric water. Once in the pre-chamber the specimens were moved to the experimental chamber (stage at ca. $-190^{\circ}C$) where they were mechanically fractured. The specimens were then etched by warming the stage. Following coating in vacuo, with gold, the specimens were viewed on a PSEM 500 (Philips, York Street,

Cambridge, England) SEM. All the equipment used in the cryogenic preparation was produced by E.M. Technology (Hexland Ltd., East Challow, England).

TABLE 1

Source of eggs used in study

		Source
Aylesbury duck	Anus platyrhynchus	6
Cape shelduck	Tadorna cana	7
Carolina duck	Aix sponsa	7
Chilean pintail	Anus georgica spinicauda	7
Coscoroba swan	Coscoroba coscoroba	7
Domestic fowl	Gallus domesticus	1, 2, 3
Great crested grebe	Podiceps cristatus	7
Guinea fowl	Numida meleagris	6
Khaki Campbell duck	Anus platyrhynchus	6
Mandarin duck	Aix galericulata	7
Ne-ne	Branta sandivicensis	7
Pink-eared duck	Malacorhynchus membranaceus	7
Red-legged partridge	Alectoris rufa	5
Ringed teal	Calonetta leucophrys	7
Ring-necked pheasant	Phasianus colchicus	5
Snake bird	Anhinga anhinga	8
South American pochard	Netta erythrophthalma	7
Domestic turkey	Meleagris gallopavo	4

TABLE 1 (cont.)

1. Commercial flock housed in batteries
2. University flock housed in batteries
3. Local retail outlets
4. British United Turkeys (Dr C. Baxter-Jones)
5. Game Conservancy Council (Dr G. V. Beer)
6. Local bird fanciers
7. Wild Fowl Trust, Slimbridge, Glos. (Prof G. V. T. Matthews)
8. Clemson University S.C. (Dr J. M. Colacino)

RESULTS

THE RESISTANCE NETWORK - STRUCTURE

CUTICLE

When first oviposited the hens' egg cuticle appears as a lustrous layer which rapidly dries (less than 30 sec in a battery unit). Electron micrographs showed that there was a concommitant change in the cuticle's fine structure from a very open granular state (Plate 2a and 2b) to the fissured, dense structure that is commonly associated with the mature cuticle (Plate 2c). The intermediate stage is shown in Plate 2d.

Substantial differences between the cuticles of white and brown eggs were observed in SEM studies (Plate 3a, 3b, 3c and 3d). The cuticles of the white eggs (Plate 3a) appeared as a delicate open structure, whereas those of brown ones (Plate 3c) were more dense. This difference was reflected in the ease with which the cuticle was removed with EDTA. That on white eggs tended to flake off whereas that on brown ones retained its integrity such that it could be removed as a sticky sheet.

The cuticle weight of brown eggs (measured by stripping the cuticle from the shell with EDTA, filtering, washing and then drying to constant weight) ranged from 0.0129 - 0.1278g per egg. The grouped frequency histogram (Fig. 3) of cuticle weight has a pronounced negative skew (-1.62, calculated according to the method described by Spiegel, 1961), with a range of 0.1 - 1.8 x $10^{-3}g/cm^2$ and a mean value of 1.27 x $10^{-3}g/cm^2$.

Fissuring was another notable feature of the cuticle (Plate 4). It was often extensive and tended to have a roughly radial orientation. There was no correlation (Fig. 4) between

crack-length and the number of underlying pores (sample of 40 eggs).

Two dyes, Green S and Tartrazine, in combination (28% and 72% respectively) make up the food dye, Edicol Supra Pea Green H. Plate 5 demonstrates the range of shades achieved by staining brown eggs (of a similar background colour) with the last mentioned. The wide range of shades has been attributed in this study to the differential retention of the two components of this stain. This was most noticeable with white eggs which only stained blue. It was noted that there was a tendency for eggs with the least cuticle weight per cm^2 of surface area (Fig. 3) to stain light green or blue whereas those cuticles staining dark green were dense in terms of weight per cm^2 of surface area. These observations suggested that fissuring as well as cuticle density influenced colour development. In order to attempt a correlation of these two, 36 brown eggs of comparable tints were stained on different sectors with the three dyes Green S, Tartrazine and Edicol Supra Pea Green H. Two sections from the shoulder region of each egg were taken for SEM (freeze dried) examination. A general trend emerged. The greater a cuticle's "crack length" (see pp 23 and Plate 4) the less Tartrazine was retained and consequently, when using Edicol Supra Pea Green H, the bluer the ultimate shade of green. When white hens' eggs were stained with Edicol Supra Pea Green H they retained only the blue component. This presumably reflected the very open nature of their cuticle (Plate 3a). Although at present the interactions between the cuticle and dyes are not fully understood, Tartrazine's lower MW 534.37 (cf Green S MW 576.63) and its greater solubility in water (140 g/l; ICI Technical Information, D1606) compared with Green S (80 g/l; ICI Technical Information, D1606) may account for these results.

Flock age affected the "quality" of the cuticle (Fig. 5). In commercial terms a good "quality" cuticle would be one that minimised bacterial and fluid penetration of the underlying pores (Smeltzer et al., 1979) and this correlates, it has been found (pp 137), with a cuticle staining in the "deep green" region (see Plate 5). It was notable therefore that the cuticle on pullets' eggs tended to stain deep green - "high quality" - whereas those on mature hens' eggs exhibited a range of colours (cf birds no. 3 and no. 8). Daily observations of 6 birds (pullets/hens) over two years showed no evidence of trends developing within a clutch or between clutches vis à vis cuticle quality. It needs to be stressed though that over this period a characteristic and uniform dye colour was displayed by all the eggs laid by several of the pullets. This indicates that quality is an inherited feature but that it diminishes with a protracted laying cycle. This is taken to be the cause of "poor" quality cuticle in old hens.

The dark porphyrin spots (see pp 8) that occasionally speckle tinted eggs only occasionally correspond to pore orifices More often they were associated with a depression in the shell's surface crystal layer (Plate 6a and 6b). This evidence indicates that such spots per se rarely, if ever, influence bacterial infection of an egg.

PORES

The geometry of the pore canal of the oviposited egg of the domestic hen is typified by Plate 7. There was no evidence in the many pores examined of material in the lumen of the canal. The

walls were of a rough texture. Material in the lumen was observed in some oviducal eggshells but this was a rare occurrence - possibly an artefact of the preparation technique.

SHELL MEMBRANES

Preparative techniques influenced the appearance of the inner and outer shell membranes. Thus a "collapsed and desiccated" appearance resulted from critical-point drying (cf Plate 8a and 8b) of membranes. Artefacts were considered to be minimal in specimens prepared by the freeze dried technique for SEM or in those prepared for TEM. Least distortion was considered to be caused by the Cryogenic method described on pp 24.

The shell membranes of all the species included in this study (Table 2) had a common structure; they were formed from either single or bundles of fibres coated with a mantle (Plate 9). A space between the mantle and core of the fibre was a characteristic feature also. The thickness (Table 2) and fibre density, however, of the inner and outer membranes differed among species (Plate 10a, 10b, 10c, 10d and 11c). The inner membrane was invariably thinner and generally contained finer fibres than the outer one. With all the membranes included in this phase of the study the void spaces between the solitary or bundles of fibres appeared to be empty. Indeed observations made in the course of the study of gas flux across the egg's integument (pp 148) supports the conclusion that the spaces are filled with air saturated with water vapour. Thus in the context of microbial infection of the underlying albumen the application of modern techniques supports a long held view that the structure of the shell membrane can be likened to

membrane type bacterial filters.

LIMITING MEMBRANE

The scanning (freeze dried and critical point dried specimens) and transmission (specimens prepared with gluteraldehyde/osmium) electron microscope were used to study the limiting membrane, which was a feature of the eggs of all the avian species included in this study (Table 2). This observation refutes the claim by Becking (1975) that the limiting membrane is a feature only of the eggs of Galliformes. Indeed, in all the eggs included in this study the limiting membrane appeared as an electron dense, smooth layer integral with the mantles of the fibres on the inner surface of the inner shell membrane (Plate 11a, 11b and 11c). There was no discernible difference in the fine structure of the limiting membrane of any of the eggs examined. Plate 11d clearly shows albumen adhering to the limiting membrane. The notable difference between the albumen and limiting membrane (cf Plate 11b and 11d) is taken to be evidence that albumen does not contribute to the formation of the latter.

DEVELOPMENT OF THE EGG IN UTERO

Duck and turkey eggs obtained from the oviducts of freshly slaughtered birds were studied with the objective of gaining further information on the occurence and nature of the limiting membrane. It was hoped to determine whether or not the limiting membrane was laid down prior to the two fibrous shell membranes or if it was formed at the same time and of the same material as the fibre mantles, which are formed in the isthmus (Plate 12). Hens' eggs could not be

included in this study because repeated attempts to get material from a slaughter line were frustrated by the managers of all the plants which I approached.

As the times taken for egg formation in the duck (Anas platyrhynchos), turkey (Meleagris gallopavo) and the hen (Gallus domesticus) are comparable, Talbot and Tyler's (1974) time-scale was applied to micrographs of 36 duck and 60 turkey eggs taken post mortem from birds slaughtered in a commercial plant.

Stages in the formation of the duck egg are represented in Plate 13 to 17. The stages in the formation of the turkey egg are very similar, therefore only the early stage (<2h) is shown (Plate 18). The times refer to the number of hours (h) since the egg first arrived in the isthmus.

In eggs that were present in the isthmus for <2h the membrane fibres (Plate 13b and 13d) appeared as three bands (thin outer, 3 - 4μm; thick middle and thin inner, 1 - 2μm), bordered on the inside (Plate 13c and 13e) by a fully developed limiting membrane and on the outside (Plate 13d) by a smooth amorphous layer. The inter-fibre space has a honey-combed appearance (Plate 13e) due to the presence of amorphous material which appeared to be similar to that of the mantles (see Plate 18c and 18e). In freeze dried samples (Plate 13b and 13c), however, the honey-combed appearance was not evident; instead strands radiated from one fibre to another. The introduction of artefacts during sample preparation is unavoidable, however, but the cryogenically prepared specimens gave probably the most accurate representation of the "soft" structures that can be achieved with current technology. This was demonstrated again with the amorphous surface layer which lies across the outer surface of the outer membrane fibres (Plate

13d). Plate 13a shows how the slower cooling of the freeze dried prepared sample resulted in the surface layer being disrupted into peaks. TEM preparations of early turkey membranes showed the interstitial material apparently fusing with the mantle of the fibres (Plate 18c and 18e).

With the onset (2h) of mineralization (Plate 14a and 14b) there was a decrease in the interstitial "honey-combing" and the membranes began to lose their trilaminate appearance (Plate 14d). It should be noted that the cryogenically prepared specimen (Plate 14d) shows the cone tips developing beneath the amorphous layer found at the outer surface of the outer membrane. This contrasts with Plate 14a. The cone layer had fused within 3h (Plate 15a), and the formation of the palisade had begun (Plate 15b and 15c). The penetration of the cone tips by membrane fibres is shown in Plate 15a.

By $5\frac{1}{2}$h the trilaminate appearance was replaced by the bilaminate structure associated with mature shell membranes, the fine inner membranes giving way to the coarser outer membrane fibres (Plate 16b). The fibres themselves were free from interstitial material (Plate 16b). Cone fusion was completed by this stage and the palisade layer was developing (Plate 16a).

After $5\frac{1}{2}$h the only discernable structural change was in shell development, the palisade layer building up to the surface crystal layer (Plate 16c and 16d). Throughout an egg's development, however, there was a significant thinning of the shell membranes (Fig. 6).

By 17h the surface crystal layer was being formed (Plate 17a). The crystal structure of this layer (Plate 17b) was notably different from that found in the palisade layer (Plate 16c and 16d).

The membrane fibres appear unchanged (Plate 17c) from those seen after $5\frac{1}{2}$h (Plate 16b) whilst the limiting membranes structure (Plate 17d) was identical to that seen at<2h (plate 13c and 13e).

The stages of turkey eggshell formation appeared very similar to those seen in the domestic duck. Transmission micrographs of thin sections (Plate 18c and 18e) indicated that the interstitial material was condensing onto the mantle of the membrane fibres. The fibres themselves were notable in that some apparently lacked a core (Plate 18b) whilst in all of them the "halo" that was associated with the core of "mature" membrane fibres was indistinct (Plate 18d).

In the context of the egg integument as a resistance network, four features of this phase of the study need to be stressed because they have been overlooked in the majority of previous studies of the microbial infection of eggs:

(1) the presence of a limiting membrane at the outset of shell formation;

(2) the material between the fibres of the immature shell membranes;

(3) the highly variable fissuring of the cuticle;

(4) the apparent rapid maturation of the cuticle following oviposition.

The present study is considered to have provided unequivocable evidence of the occurrence of a limiting membrane and subsequent studies were concerned with defining its contribution, if any, to (a) gas flux across the egg's integument (pp 148), and (b) the impediment of microbial invasion of the underlying albumen (pp 155). If the shell membranes are considered in the context of "bacterial filters", then this resistance to bacterial invasion will be marked in the immature (oviducal) stage because of the granular

material between the fibres. Indeed, this may have adaptive significance in protecting the albumen from infection before the calcitic shell is laid down, it being well documented (Romanoff and Romanoff, 1949) that foreign objects and microorganisms may be forced along the oviduct by reverse peristalsis. This topic was not examined further. The third feature, the extent of fissuring of the cuticle, might be expected to compromise the cuticle's protection of the underlying pore canals from bacterial invasion, a topic discussed on pp 253 in the context of bacterial infection of eggs exposed to the unsatisfactory features (warm eggs:cold water) found in poorly supervised egg washing. Indeed, the novel observations on a rapid change in the cuticle post-oviposition led to a limited study (pp 134) the results of which suggest that future studies of salmonellae transmission by hatching eggs ought to concentrate on husbandry factors that favour/hinder bacterial penetration of eggs in the nest boxes or floor litter, it being assumed that little benefit would be obtained from washing eggs that had already been invaded by bacteria.

TABLE 2

Occurrence of the limiting membrane and shell membrane thickness

	n	Mean (and SD) shell membrane thickness (μm)*	Limiting membrane present/absent
Aylesbury duck	12	90 (2.49)	+
Cape shelduck	4	125 (4.11)	+
Carolina duck	7	80 (2.56)	+
Chilean pintail	10	64 (5.40)	+
Coscoroba swan	7	78 (11.32)	+
Domestic fowl	40	70 (3.21)	+
Great crested grebe	1	70	+
Guinea fowl	2	65 (8.48)	+
Khaki campbell	8	81 (3.10)	+
Mandarin duck	5	77 (1.13)	+
Ne-ne	7	120 (4.12)	+
Pink-eared duck	8	67 (1.60)	+
Red-legged partridge	2	68 (7.07)	+
Ringed teal	3	65 (2.51)	+
Ring-necked pheasant	4	65 (2.21)	+
South American pochard	6	88 (1.79)	+
Domestic turkey	10	110 (3.00)	+

* Measurements were taken from Transmission electron micrographs.

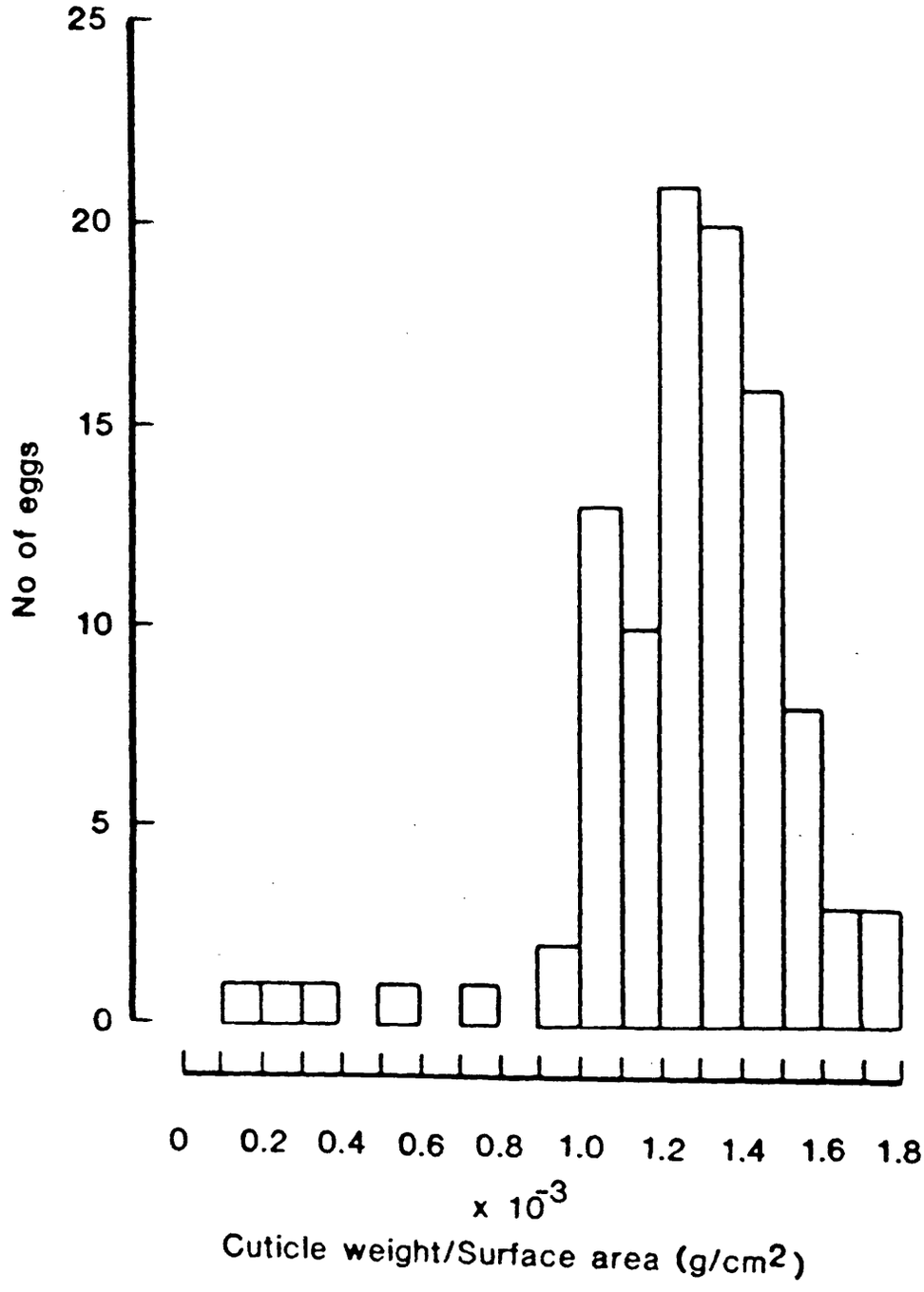


Fig. 3 The range and distribution of the cuticle on brown eggs. The cuticle was stripped from the egg with neutralised ethylenediaminetetraacetic acid (EDTA) and harvested on a preweighed glass microfibre filter.

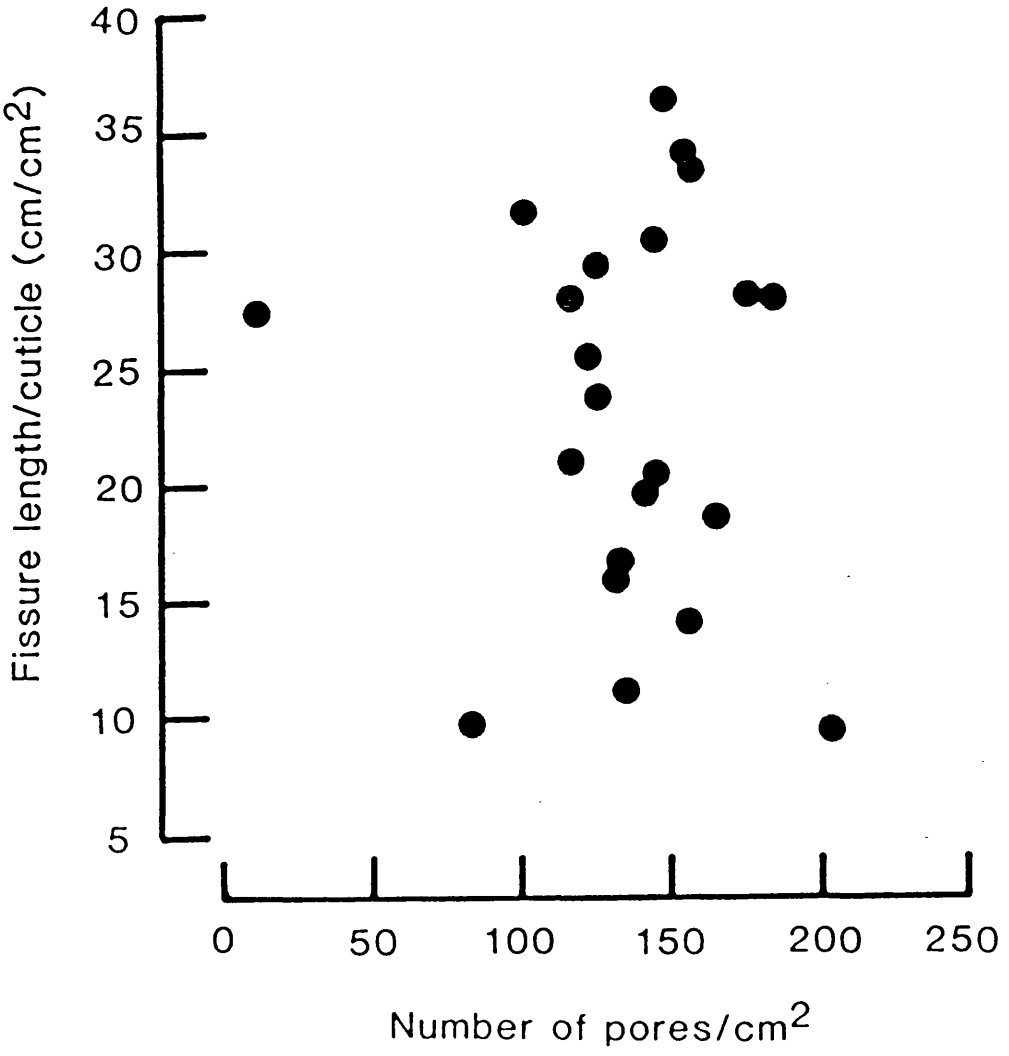


Fig. 4 The extent of fissuring in the cuticle on brown hen eggs was not related to the number of underlying pores.

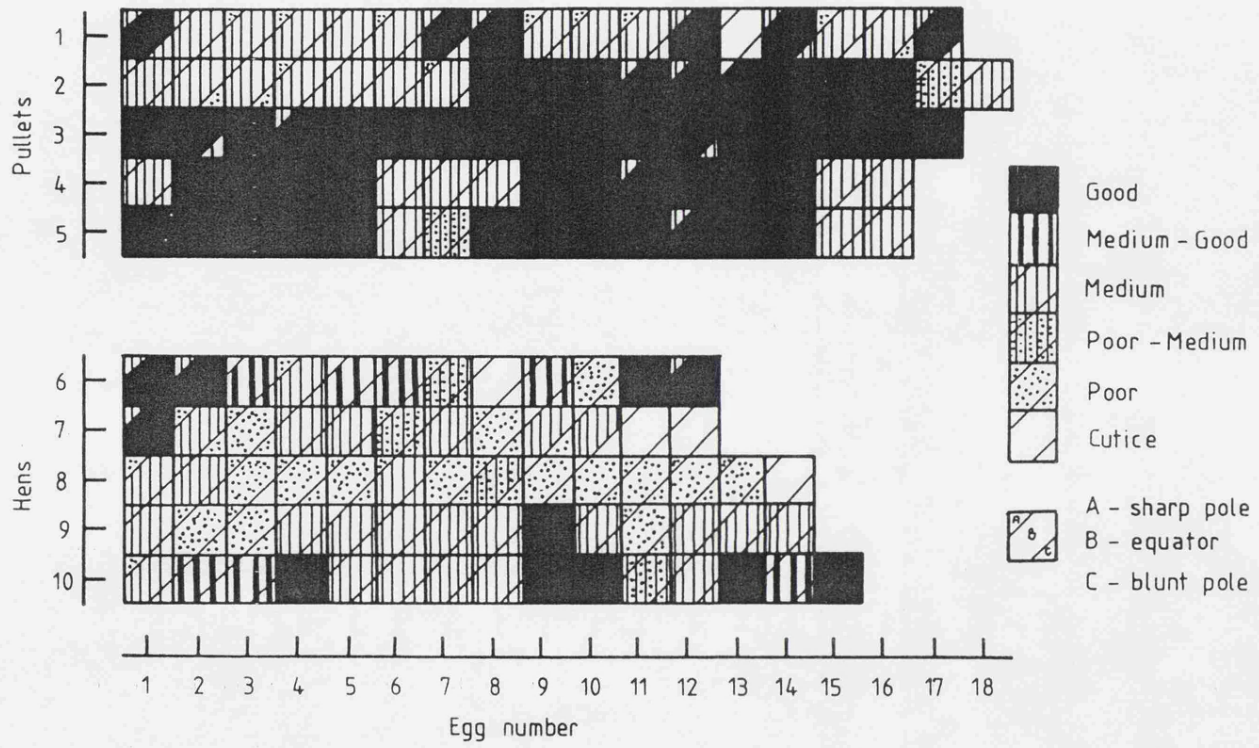


Fig. 5 Diagramatic representation of cuticle quality as determined by staining with Edicol Supra Pea Green H.

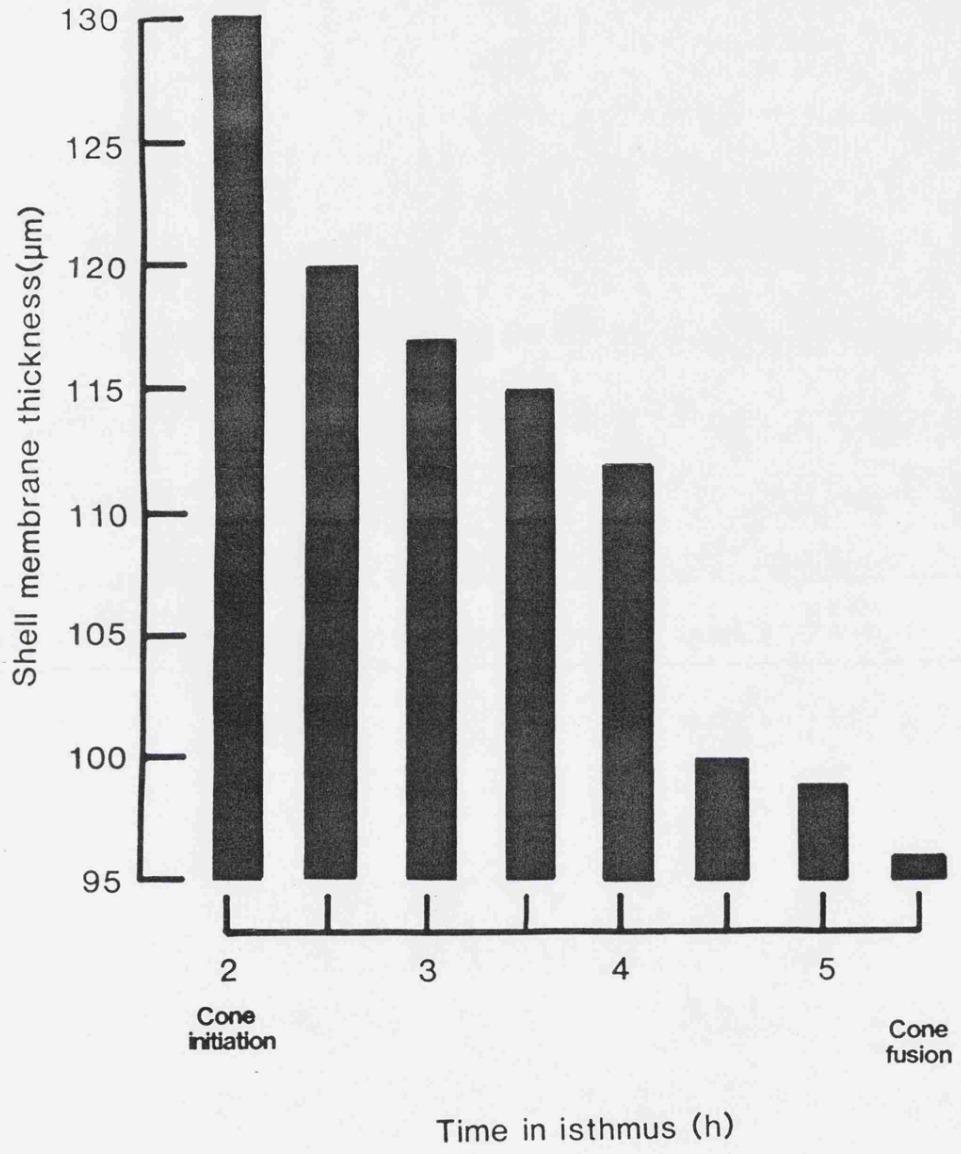


Fig. 6 The thickness of the immature shell membranes diminished as plumping fluid entered the egg prior to cone fusion.

PLATE 2.

a) The open "frothy" structure of the immature cuticle on a brown hen's egg immediately post-oviposition.
Bar marker 10μm.

b) Immature cuticle - cf mature cuticle (Plate 2c).
Bar marker 100μm.

c) Mature cuticle on brown hen's egg.
Bar marker 100μm.

d) The intermediate stage of cuticle maturity showing the development of the "plaque" structure.
Bar merker 100μm.

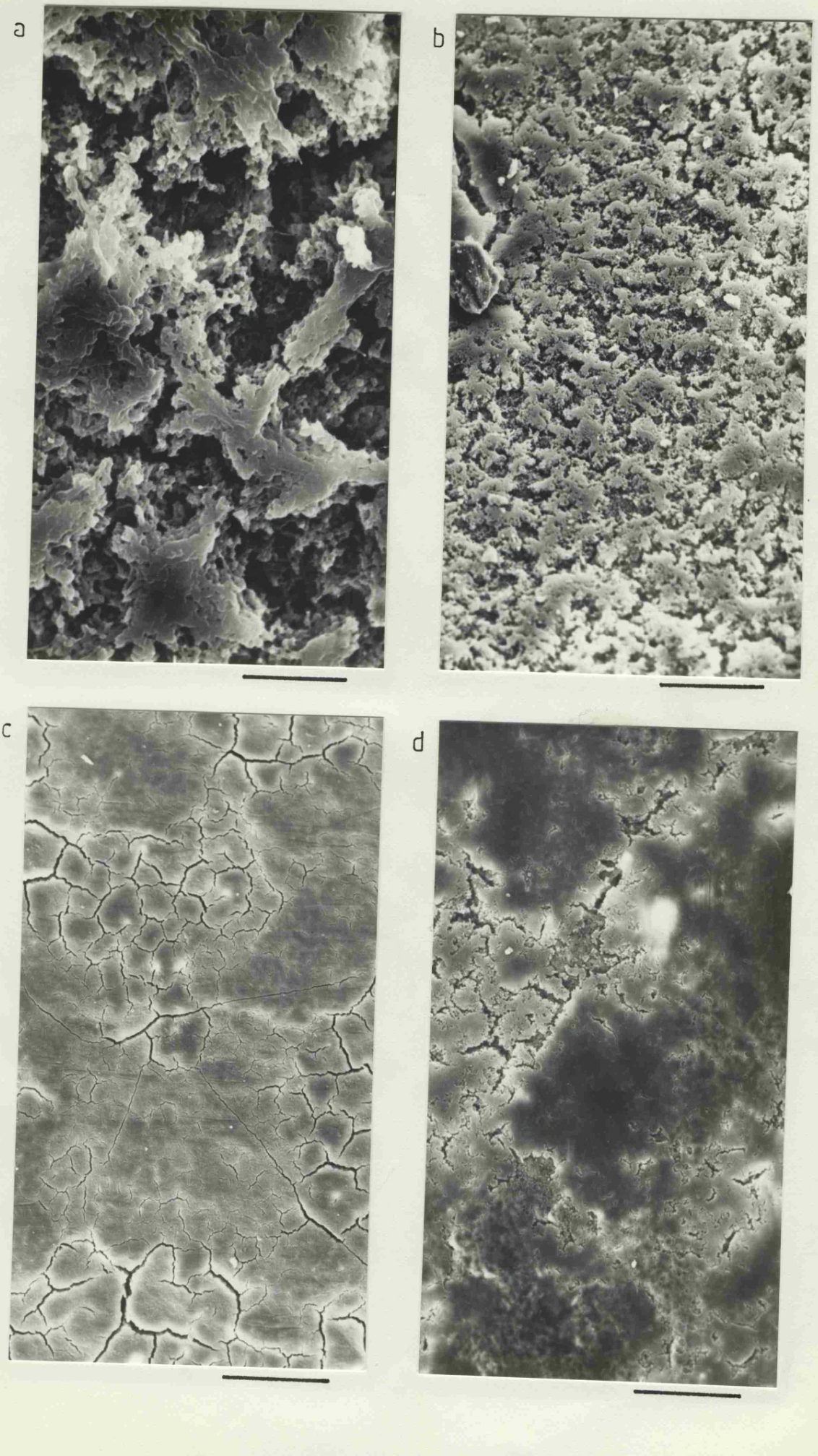

a
b
c
d

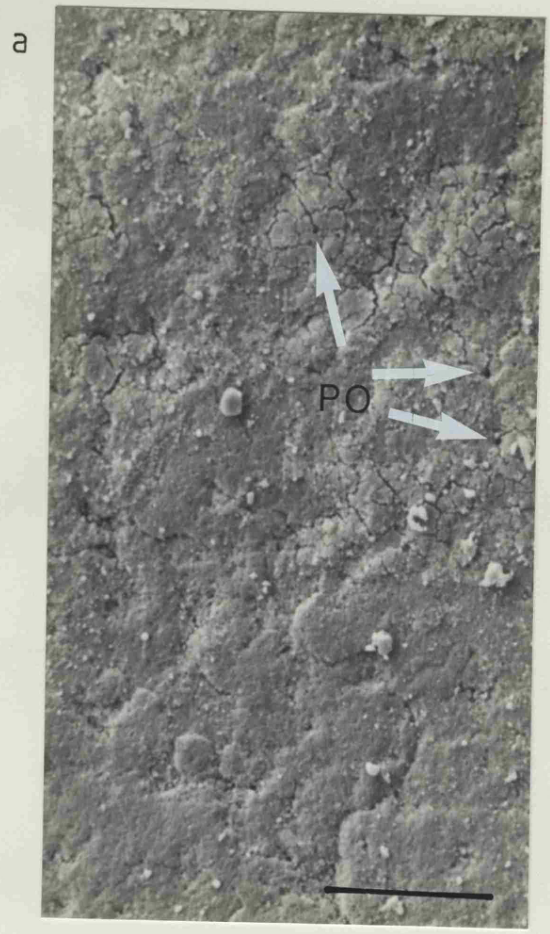
PLATE 3.

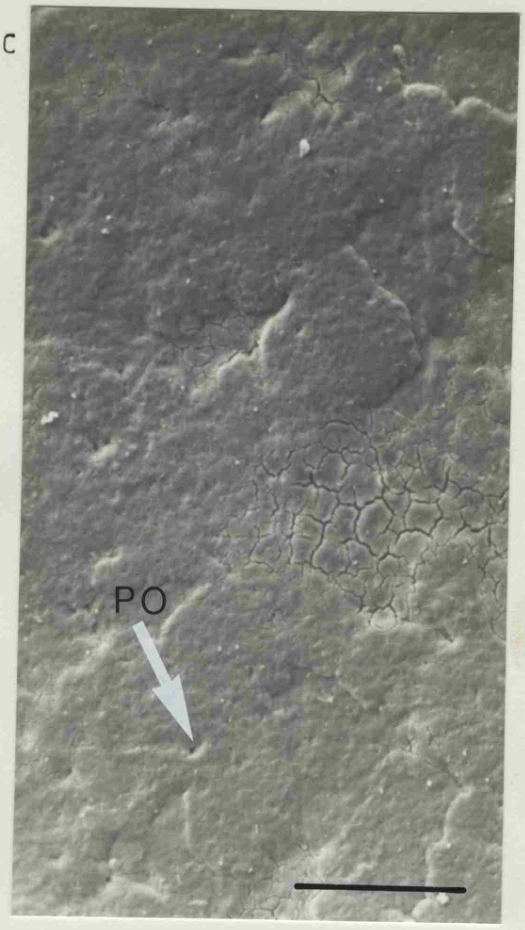
a) Micrograph of the cuticle on a hen's white eggshell. Numerous unobstructed pore orifices (PO) are visible. Bar marker 100µm.

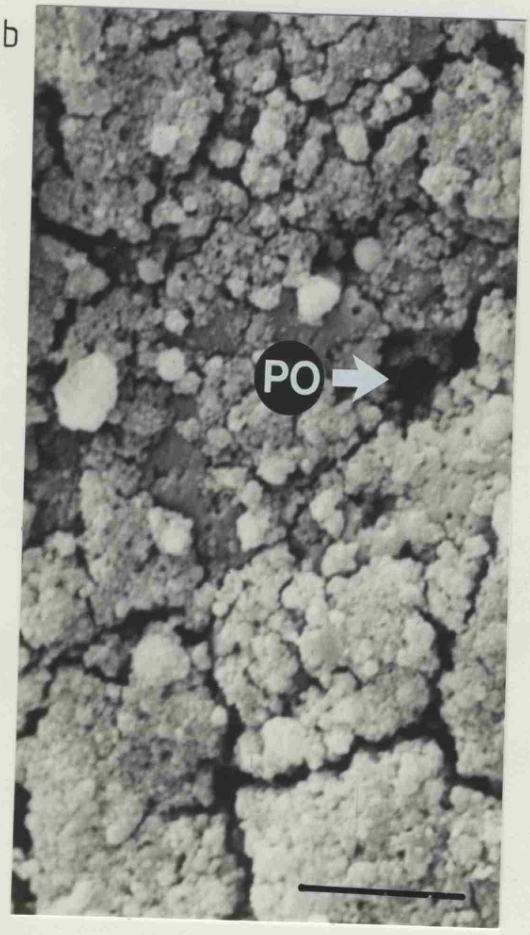
c) Micrograph of the cuticle on a hen's brown eggshell showing an unobstructed pore orifice (PO). Bar marker 100µm.

b) Close up of Plate 3a. The pore orifice (PO) is surrounded by the typically open granulated cuticle. Bar marker 10µm.

d) Close up of Plate 3c. The cuticle is quite distinct from that in Plate 3b. Bar marker 10µm.


a
PO


c
PO


b
PO


d

PLATE 4.

Scanning electron micrograph of a hen's brown eggshell cuticle showing plaques (P) overlaying fissures (F).
Bar marker 100μm.


P
F

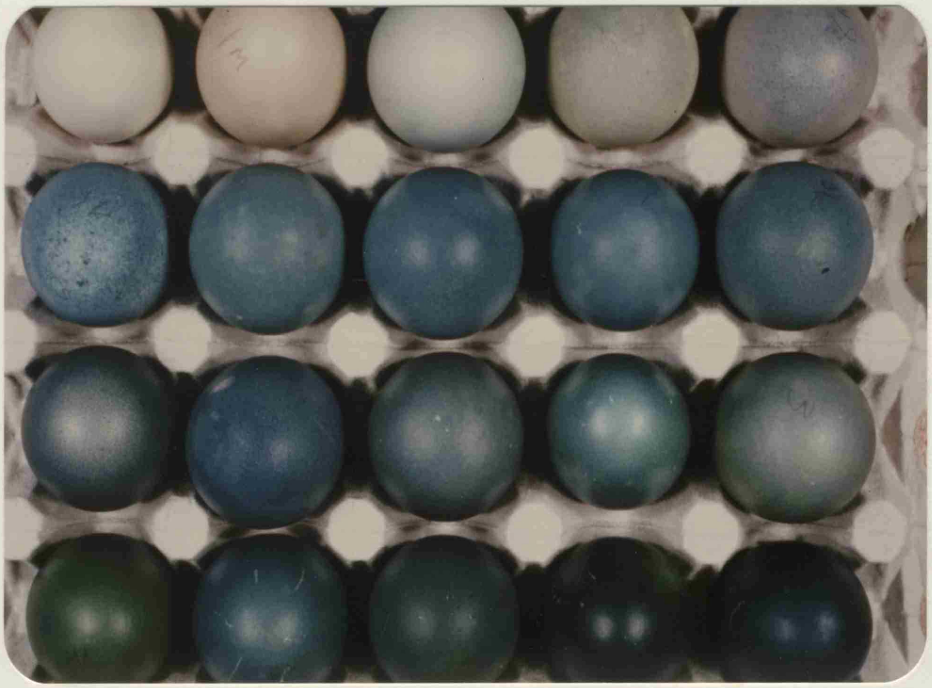


PLATE 5. Range of tints achieved by staining the cuticle of brown eggs with the dye Edicol Supra Pea Green H.

PLATE 6(a). The cuticle on a brown hen's egg showing the dark porphyrin spots. The shell has been scored to provide a reference point.

(b). Removing the cuticle (with EDTA) revealed the underlying pore orifices. Although porphyrin spots are almost always associated with an opening the opposite does not apply, therefore the degree of porphyrin speckling does not relate to pore number.

PLATE 7.

Radial section of hen eggshell showing the diffusion pathway: cuticle (C), pore canal (PC), cone layer (CL) and shell membranes (SM). Bar marker 100μm.

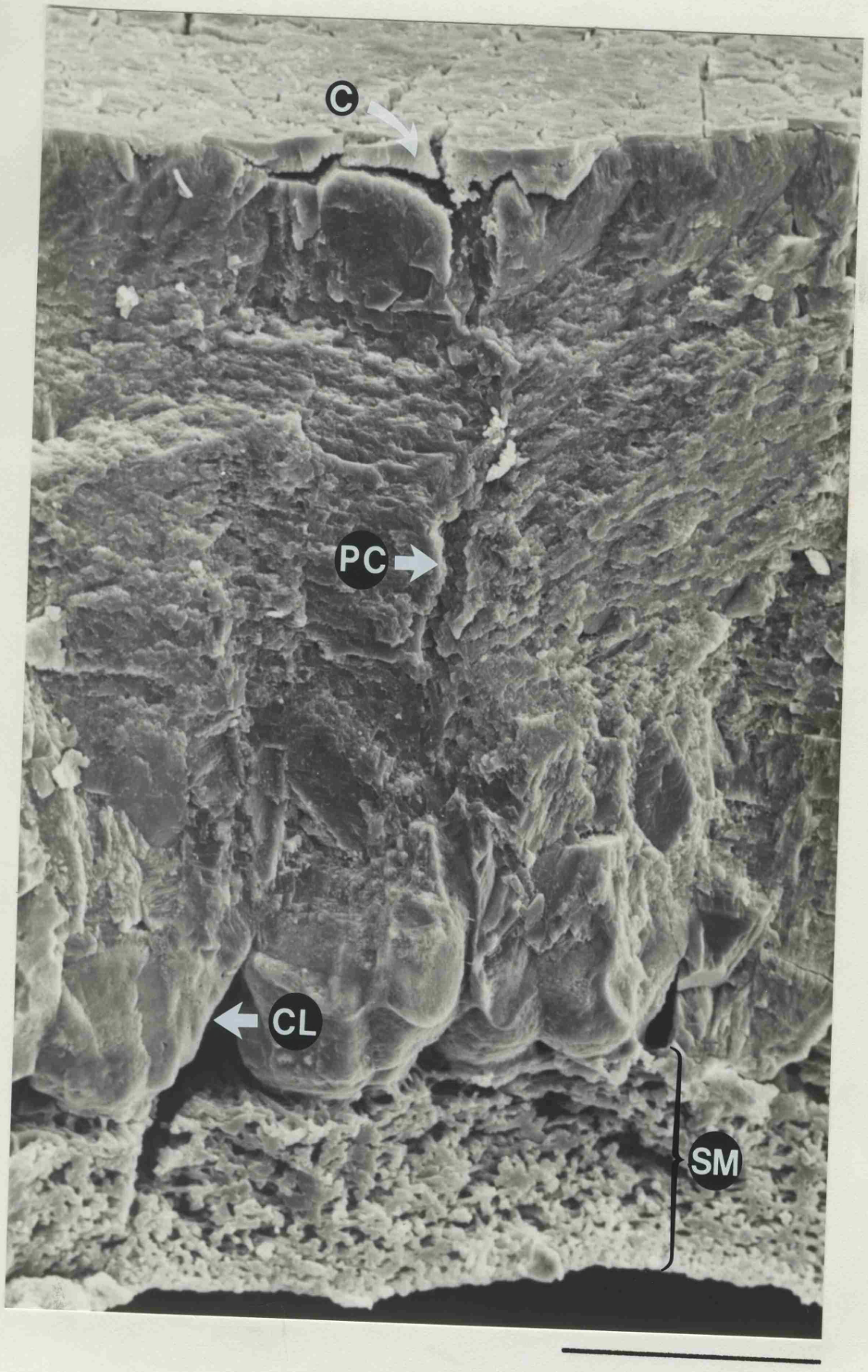

C
PC
CL
SM

PLATE 8.

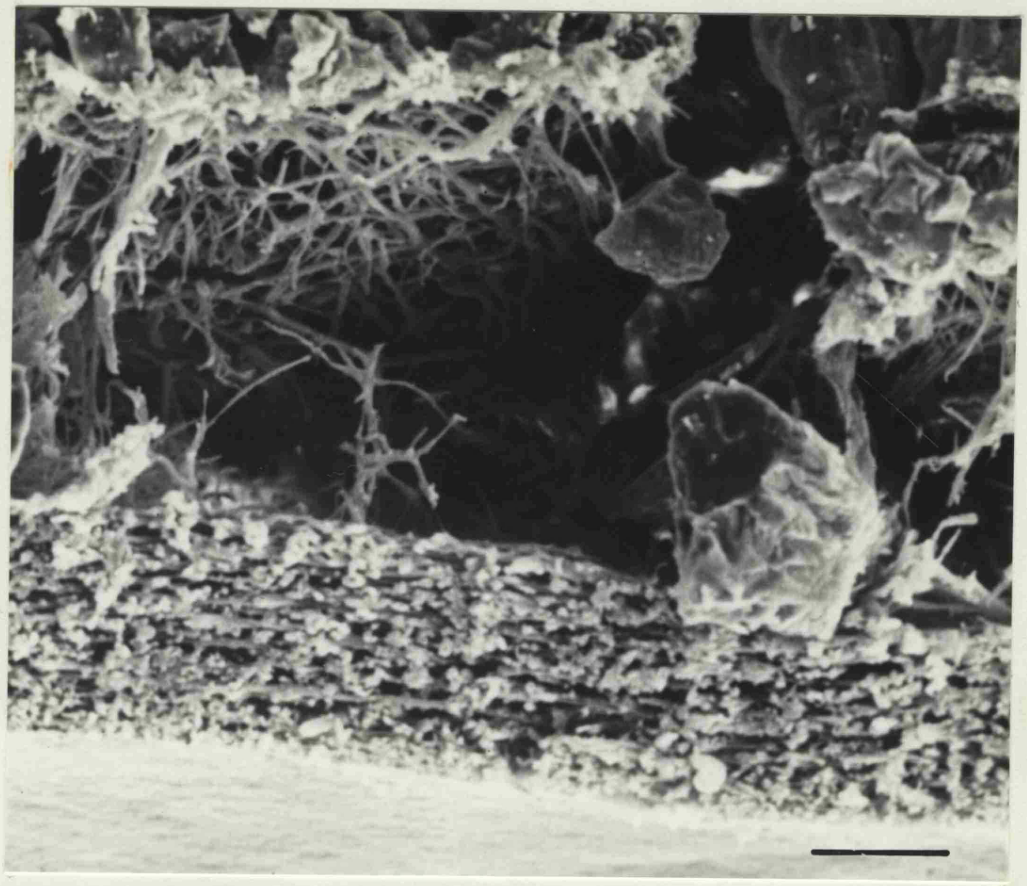
a) Shell membranes prepared for SEM by critical point drying have a collapsed and dessicated appearance.

Bar marker 10μm.

b) Shell membranes prepared for SEM by freeze drying.

Bar marker 10μm.

a



b

PLATE 9

Transverse section of a hen eggshell outer membrane fibre showing a common mantle (M), the individual fibre cores (C) and the interfibrillar space (IS).

Bar marker 1μm.

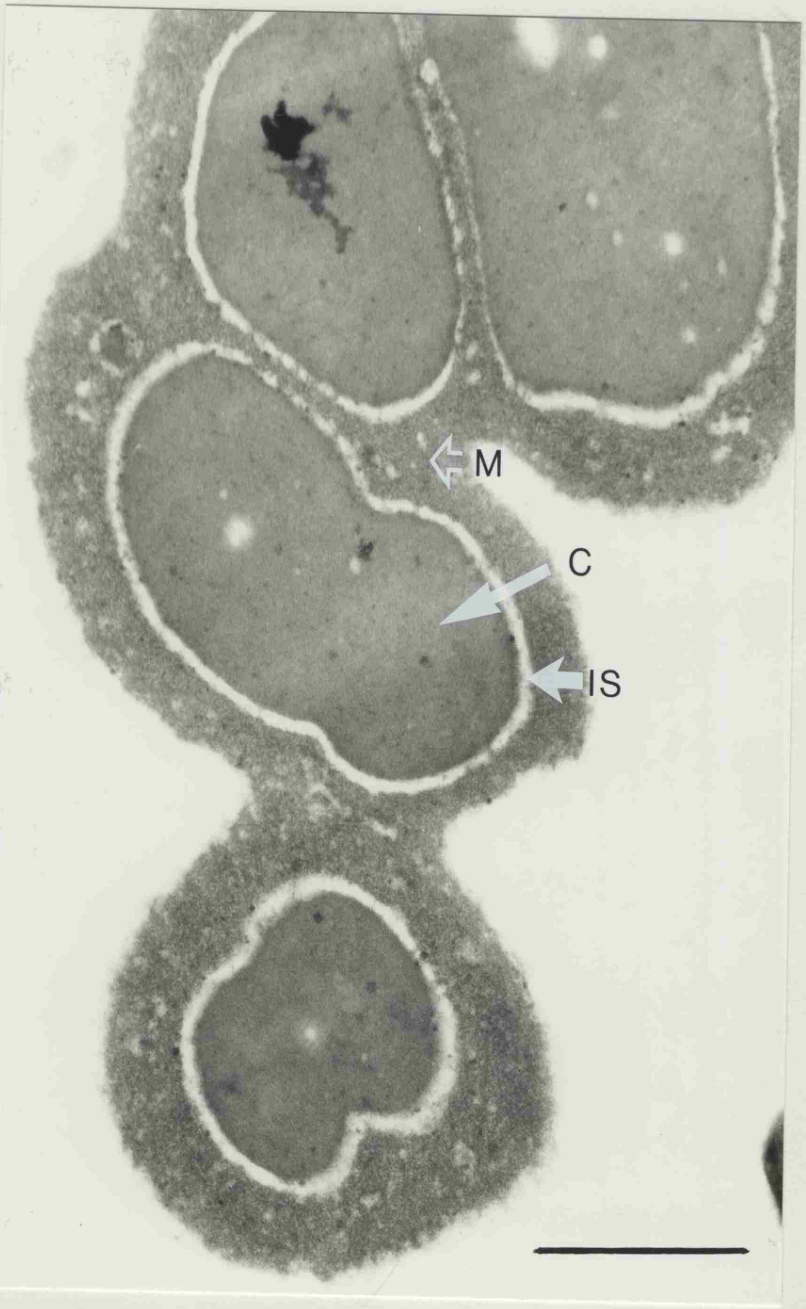

M
C
IS

PLATE 10

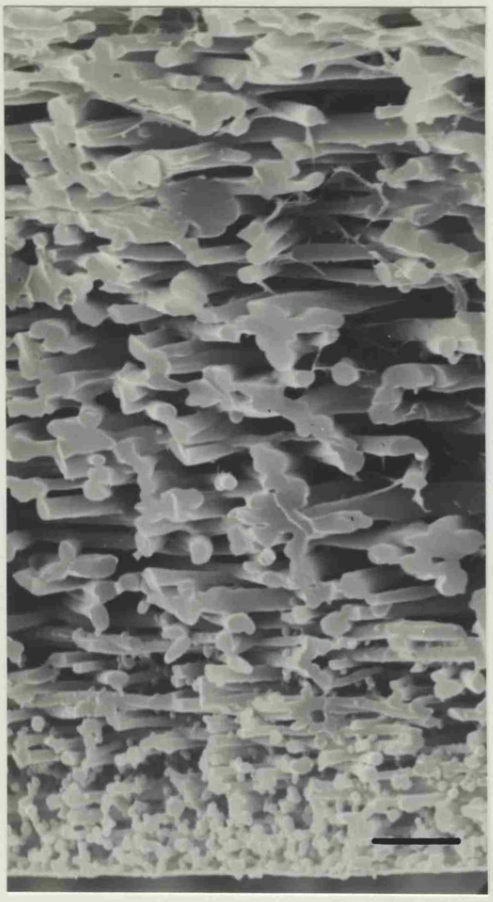
a) Radial section of Chilean pintail eggshell membranes. Bar marker 10μm.

c) Radial section of Mandarin duck eggshell membranes. Bar marker 10μm.

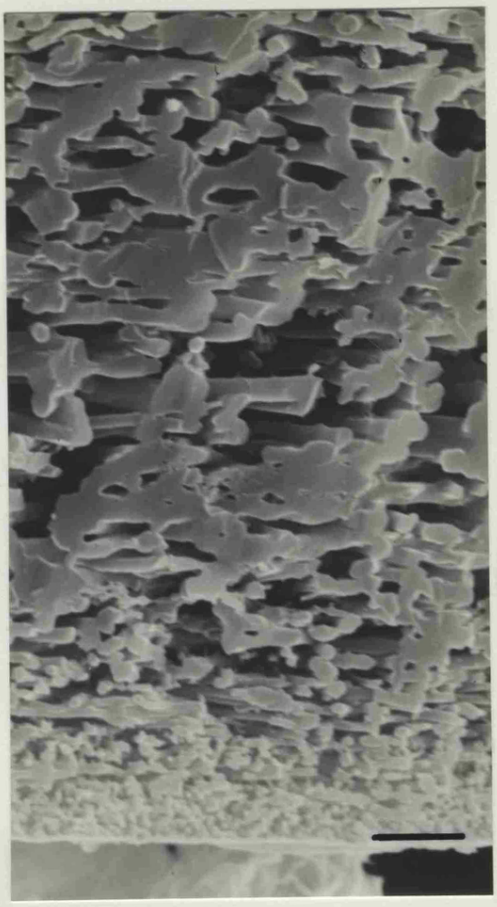
b) Radial section of South American pochard eggshell membranes. Bar marker 10μm.

d) Radial section of Carolina duck eggshell membranes. Bar marker 10μm.

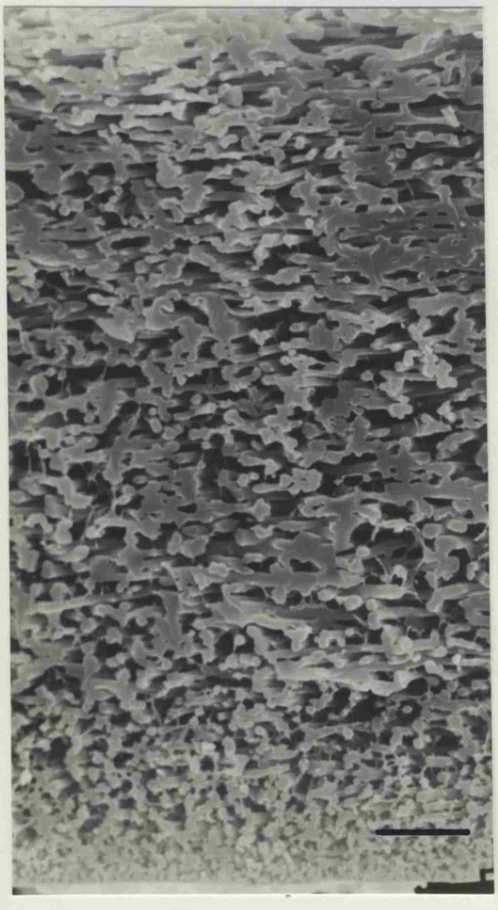
a



c



b



d

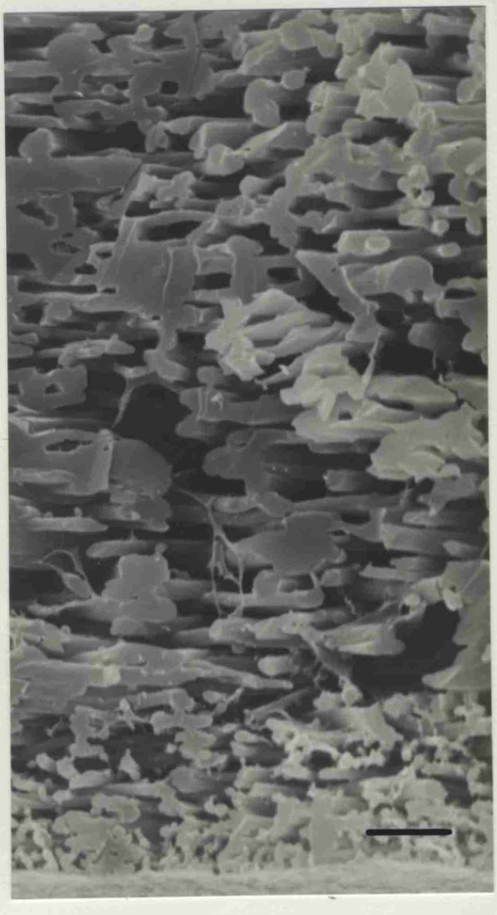


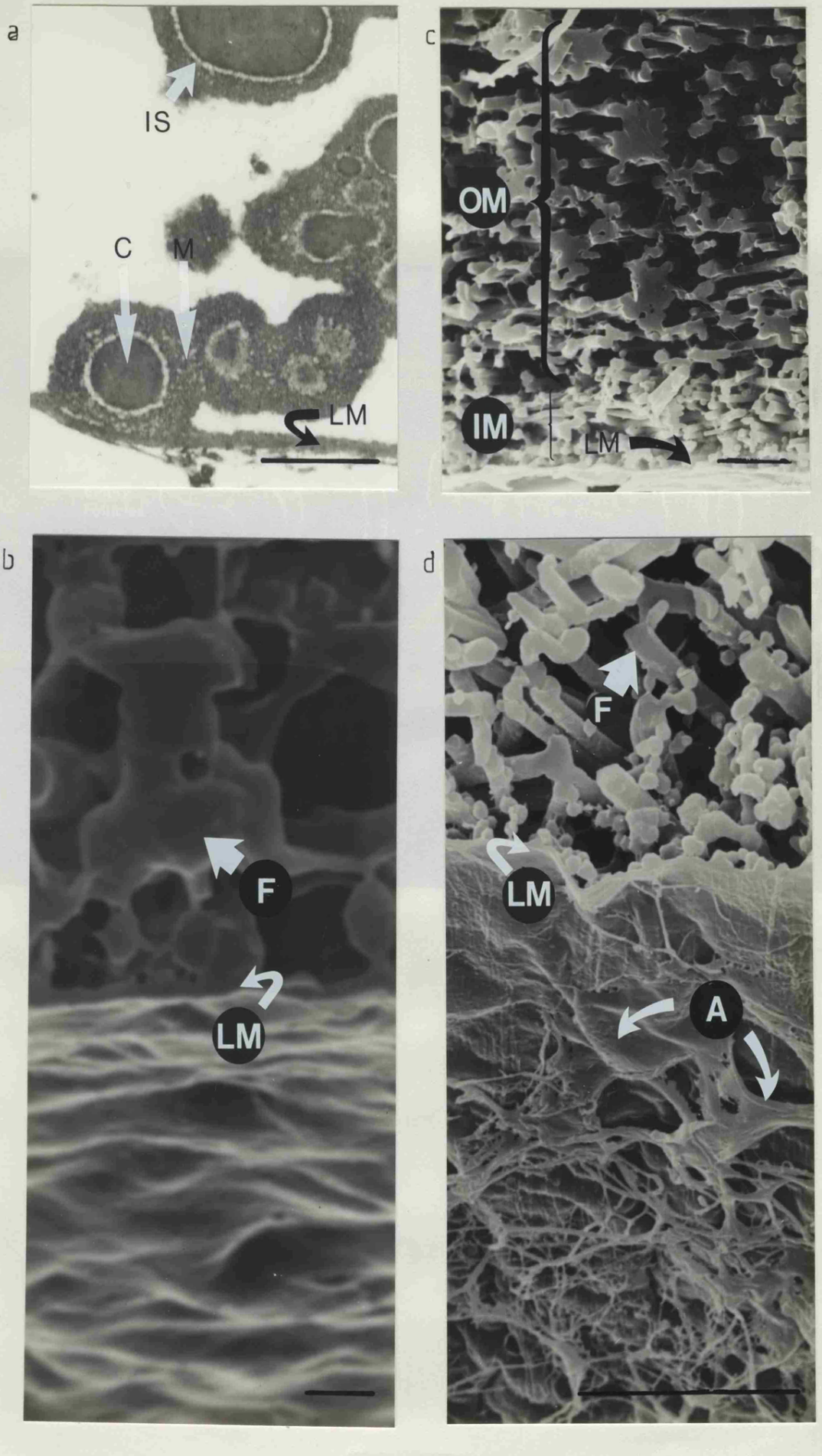
PLATE 11

a) Electron micrograph of a transverse section of the limiting membrane (LM) and inner membrane fibres showing the core (C), mantle (M) and interfibrillar space (IS).
Bar marker 1μm.

b) Radial section of the inner membrane fibres (F) and the limiting membrane (LM) from Aylesbury duck eggs.
Bar marker 1μm.

c) Radial section of domestic hen's eggshell membrane showing the outer (OM), inner (IM) and limiting membranes (LM).
Bar marker 10μm.

d) Radial section of the inner membrane fibres (F) and the limiting membrane (LM) showing albumen (A) adhering to the limiting membrane.
Bar marker 10μm.


a
IS
C
M
LM
c
OM
IM
LM
b
F
LM
d
F
LM
A

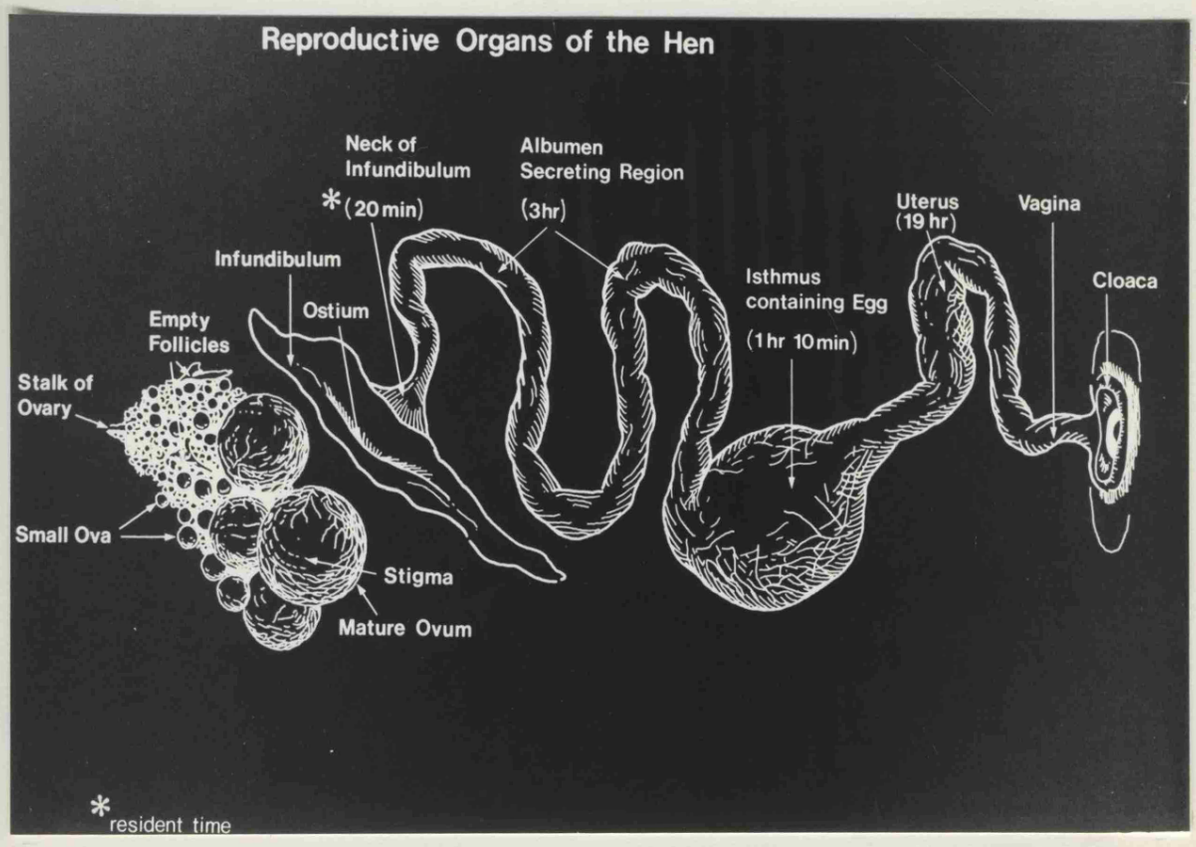

Reproductive Organs of the Hen
Neck of Infundibulum
*(20 min)
Albumen Secreting Region
(3hr)
Uterus (19 hr)
Vagina
Cloaca
Infundibulum
Ostium
Isthmus containing Egg
(1 hr 10 min)
Empty Follicles
Stalk of Ovary
Small Ova
Stigma
Mature Ovum
*resident time

PLATE 12.

PLATE 13.

a) Duck egg (<2h). Surface view of the outer surface of the outer membrane showing the peaks of amorphous material (AM) overlying the fibres (F). There is no evidence of shell initiation. Bar marker 100μm.

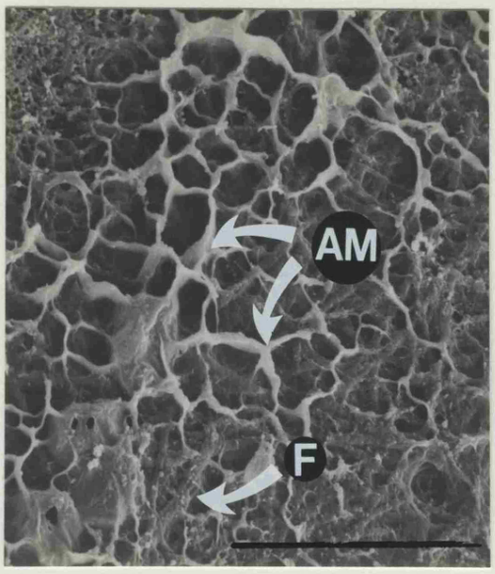
b) Duck egg (<2h). Radial section of egg membrane showing the outer (OM), inner (IM) and limiting membrane (LM). Fine threads of an amorphous material can be seen linking the membrane fibres. Bar marker 10μm.

c) Duck egg (<2h). Radial section through the limiting membrane (LM), the inner fibres (F) of the inner membrane and the amorphous material (AM) linking them. Bar marker 1μm.

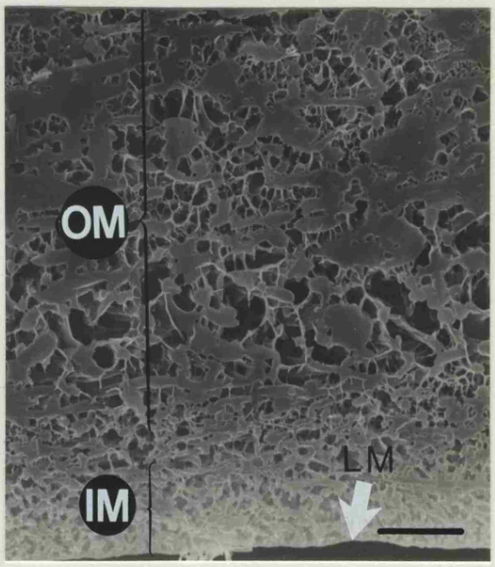
e) Duck egg (<2h). Close up of the section shown in Plate 13d. The limiting membrane (LM) is clearly visible as are the inner membrane fibres (F) and the interstitial granular material that links them. Bar marker 10μm.

d) Duck eggs (<2h). Radial section through an early shell integument prepared by cryogenic technique (cf Plate 13b - prepared by freeze drying). Note the trilaminate appearance of the membrane fibres and the associated interstitial granular material (IM) linking the individual fibres (F). The amorphous material (AM) on the outer surface of the outer membrane appear as a discrete layer (cf Plate 13a - prepared by freeze drying). Bar marker 10μm.

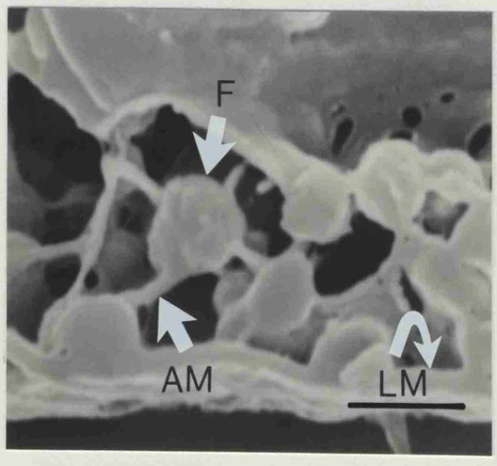
a



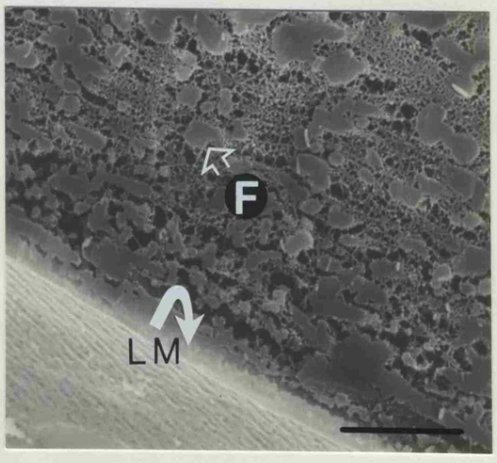
b



c



e



d

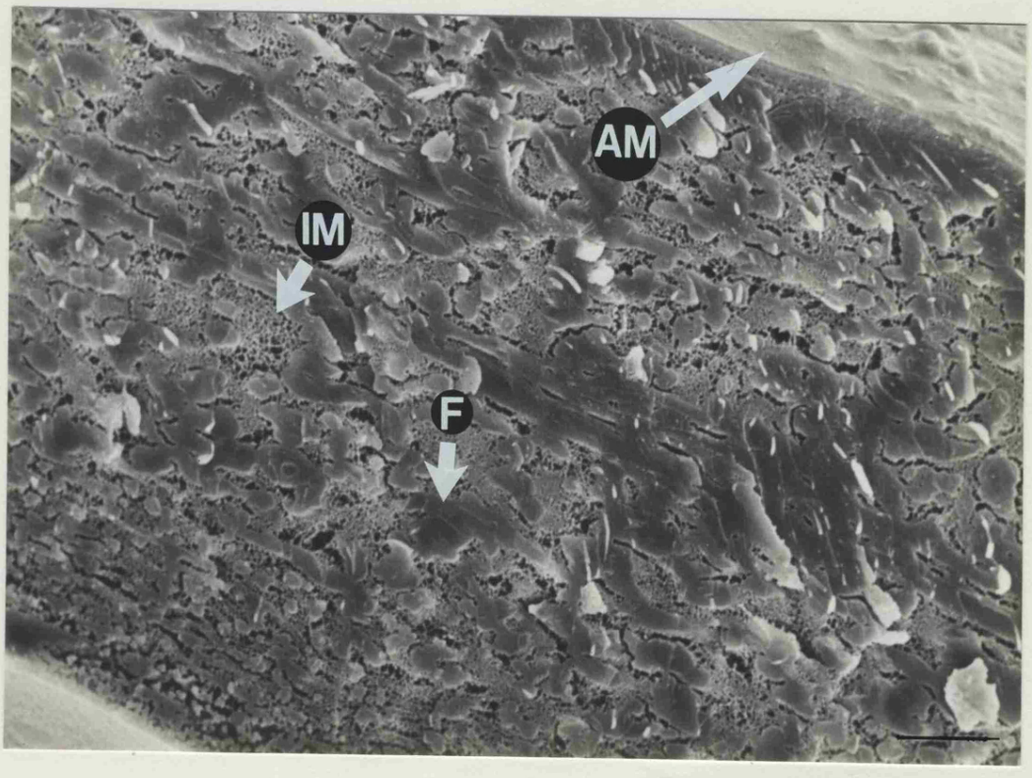


PLATE 14.

a) Duck egg (2h). Outer surface of the outer membrane showing developing cones (C) and the underlying outer membrane fibres (F).
Bar marker 100μm.

b) Duck egg (2h). Developing cone tip.
Bar marker 10μm.

c) Duck egg (2h). Radial section through the outer, inner and limiting membrane. Note the decrease (cf Plate 13b) in interstitial material (IM).
Bar marker 10μm.

d) Duck egg (2h). Cryogenic preparation showing the developing cone tips (C) surrounded by amorphous material (AM).
Bar marker 10μm.

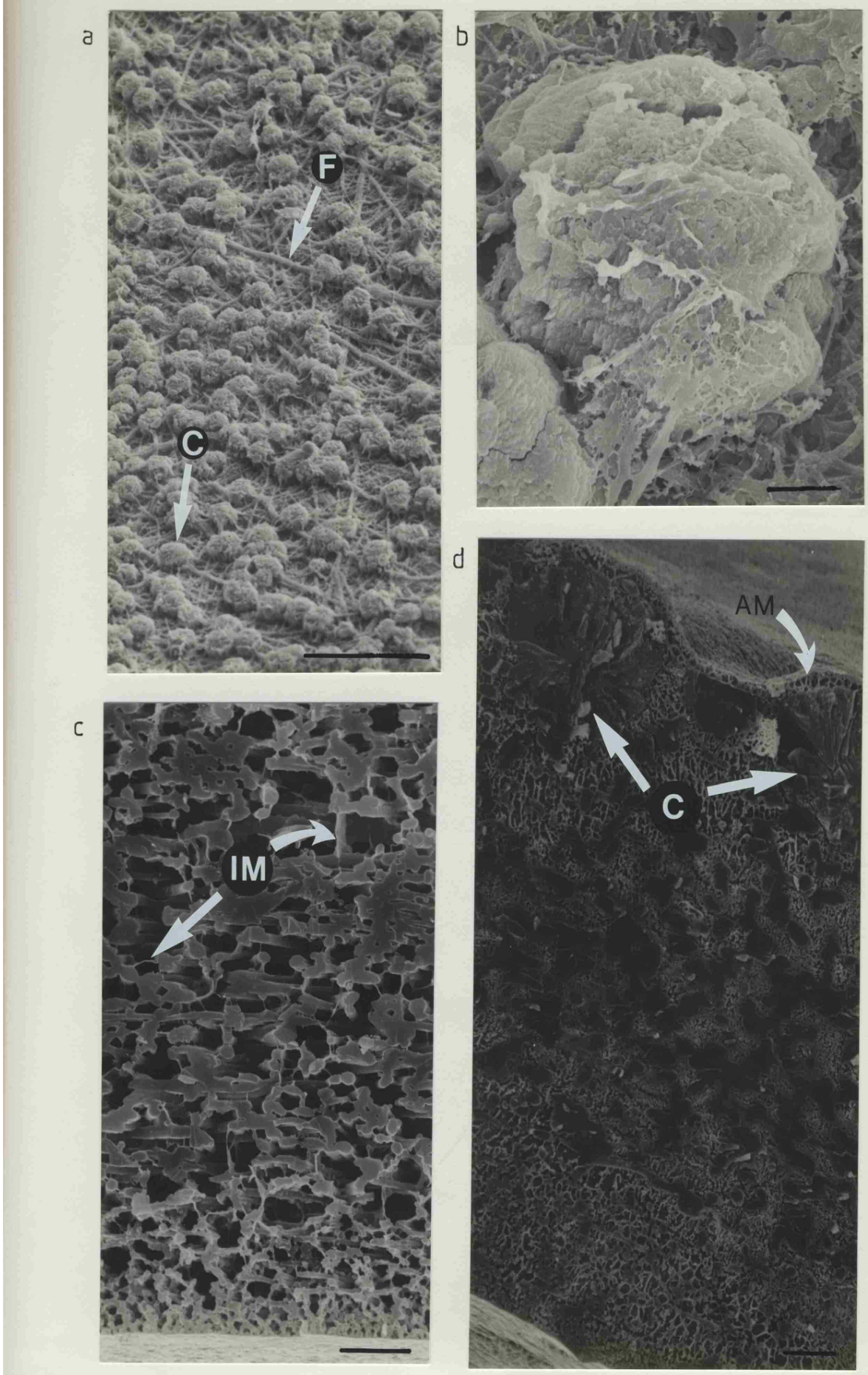

a
F
C
b
c
IM
d
AM
C

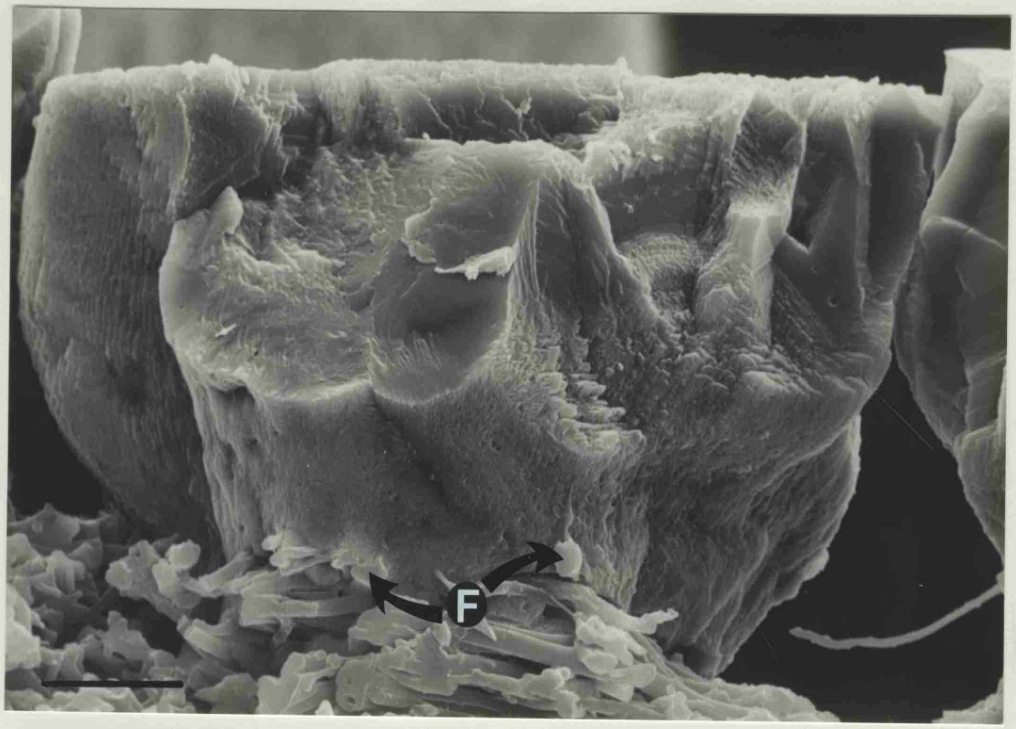
PLATE 15.

a) Duck egg (3h). Radial view of a cone tip at the point of fusion with its neighbours. The outer membrane fibres (F) can be seen entering the tip.
Bar marker 10μm.

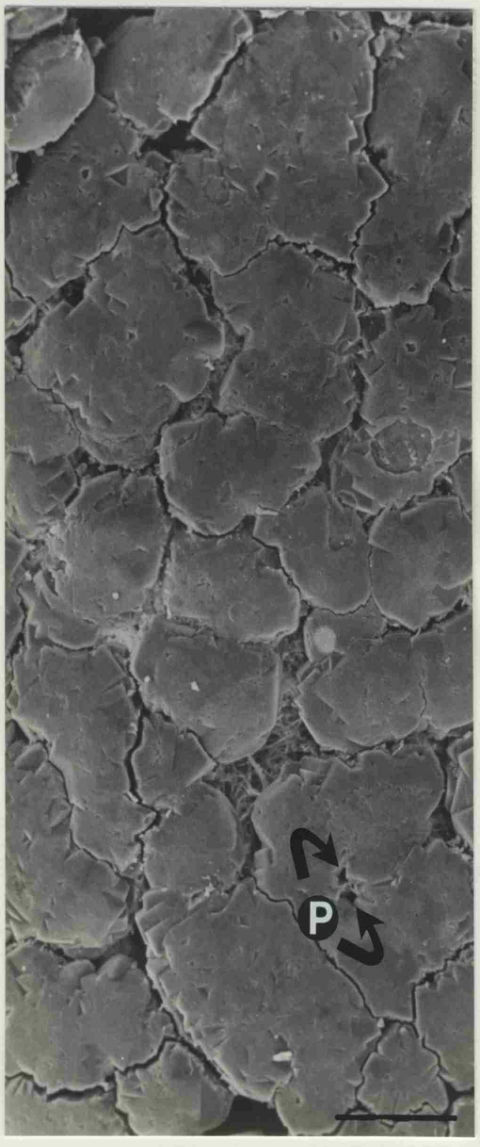
b) Duck egg (4h). Surface view of the cone layer; the individual cones are begining to merge and pores (P) are being initiated.
Bar marker 100μm.

c) Duck egg (4h). Radial section through shell membrane and cone layer. The cones (C) have fused.
Bar marker 10μm.

a



b



c

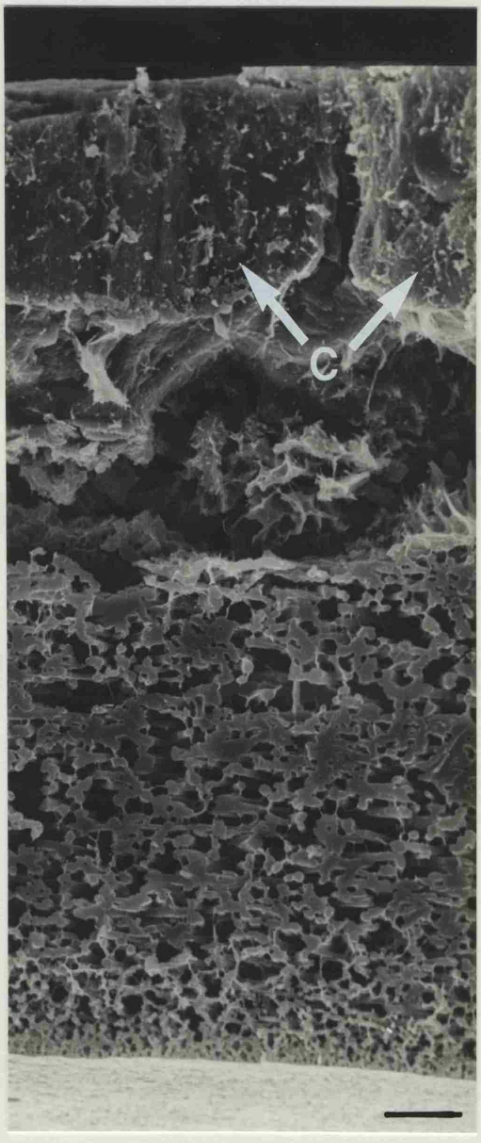


PLATE 16.

a) Duck egg ($5\frac{1}{2}$h). Radial section through the cone layer. The point of fusion (F) between the neighbouring cone tips is clearly visible as are the concave faces (C) due to the removal of a neighbouring tip.
Bar marker 10μm.

b) Duck egg ($5\frac{1}{2}$h). Radial section through the shell membranes. Note the loss of the trilaminate appearance (cf Plate 13b), fibre aggregates and the amorphous material linking the fibres.
Bar marker 10μm.

c) Duck egg (4-5h). Surface view of developing shell showing sheet-like crystal development.
Bar marker 10μm.

d) Duck egg (4-5h). Twin pore canals (PC) in the surface of the advancing shell face.
Bar marker 10μm.

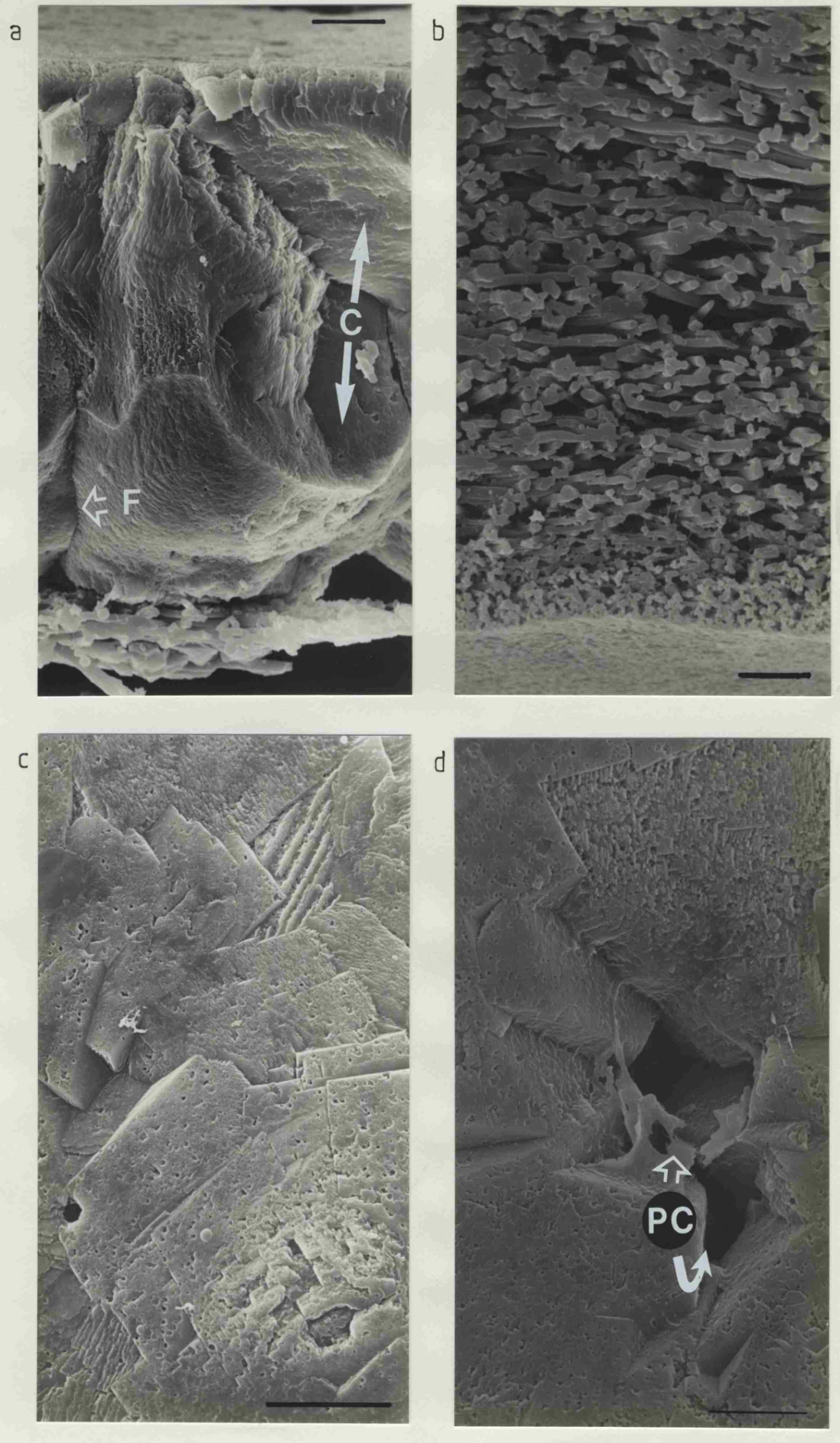

a
C
F
b
c
d
PC

PLATE 17.

a) Duck egg (17h). Radial section of an almost complete shell integument showing the shell membranes (SM), cone tips (CT), palisade layer (PL) and surface crystal layer (SC).
Bar marker 100μm.

b) Duck egg (17h). Surface view of the surface crystal layer. Note the difference in crystal type cf Plate 16c.
Bar marker 10μm.

c) Duck egg (17h). Radial section through shell membranes (cf Plate 13b and 14d).
Bar marker 10μm.

d) Duck egg (17h). Inner membrane fibres (F) with attendant nodules (N). The limiting membrane appears unchanged from that in Plate 13c.
Bar marker 1μm.

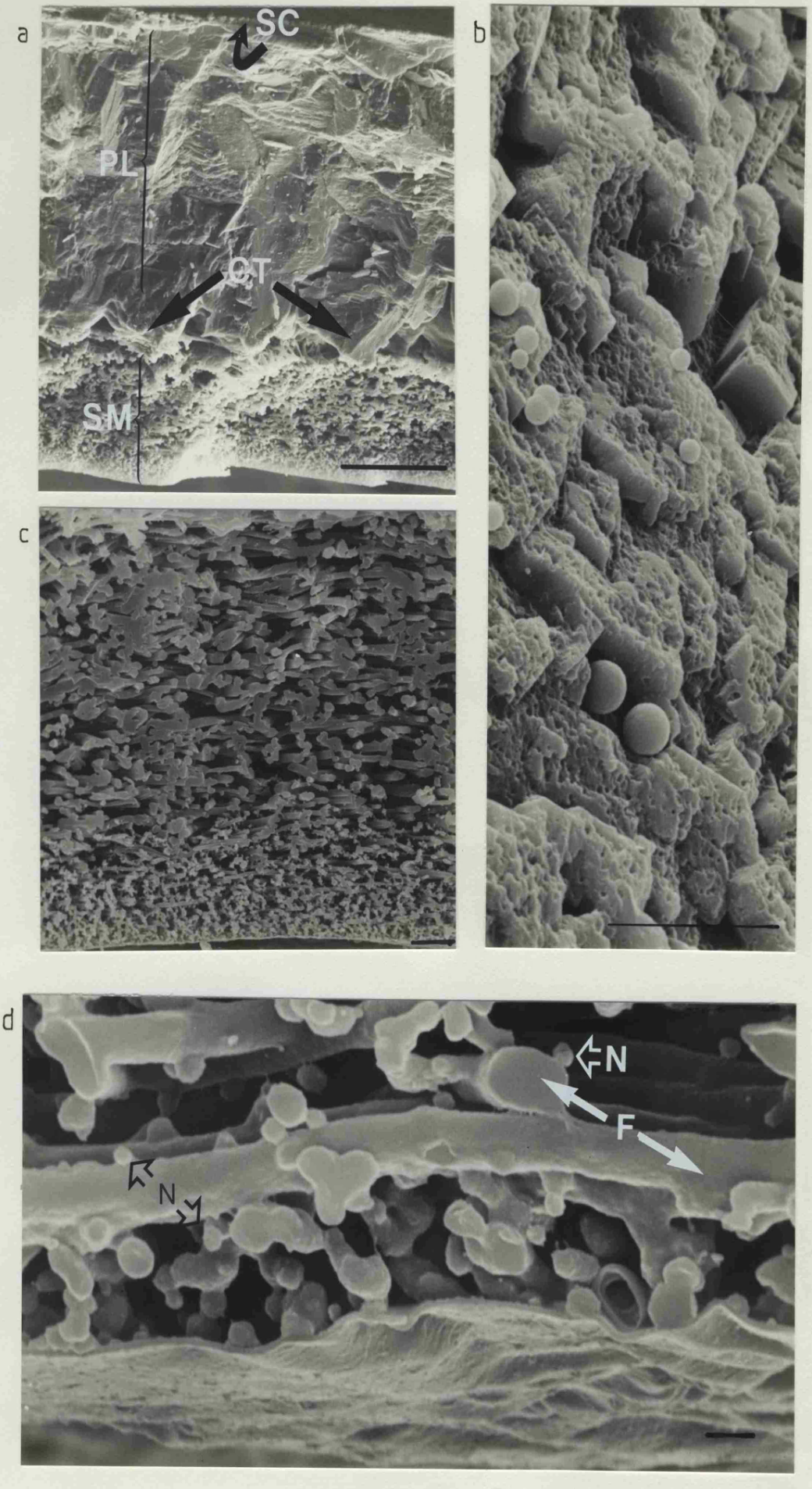

a
SC
PL
CT
SM
b
c
d
N
F
N

PLATE 18.

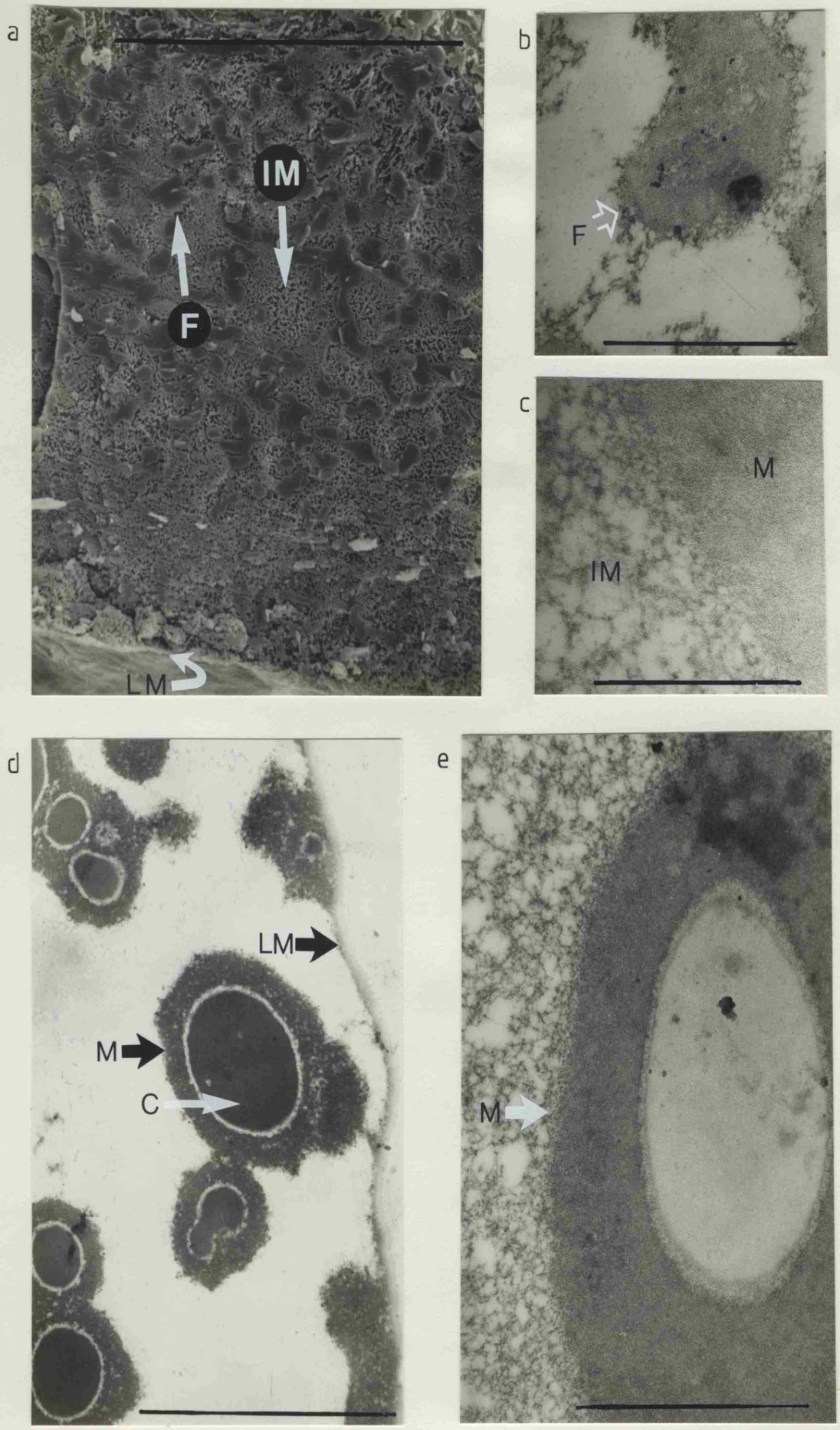
a) Turkey egg (<2h). Transverse section through cryogenically prepared shell membranes. The limiting membrane (LM) appears fully formed. The fibres (F) are linked by interstitial material (IM).
Bar marker 100μm.

b) Turkey egg (<2h). TEM section through an inner shell membrane fibre. Note the lack of a core (cf Plate 18d, e).
Bar marker 1μm.

c) Turkey egg (<2h). Close up of Plate 18e showing interstitial material (IM) apparently condensing onto the fibre mantle (M).
Bar marker 1μm.

d) Turkey egg (oviposited) TEM section through the limiting membrane (LM) and inner membrane fibres. The core (C) of the fibre is separated from the mantle (M) by a clear zone.
Bar marker 10μm.

e) Turkey egg (<2h). TEM section appearing to show interstitial material condensing onto the mantle (M) of an inner membrane fibre. Note Ruthenium red was not used to prepare this section hence the light appearance of the core.
Bar marker 1μm.


a
IM
F
LM
b
F
c
M
IM
d
LM
M
C
e
M

LITERATURE REVIEW

THE RESISTANCE NETWORK - FUNCTION

LITERATURE REVIEW

THE RESISTANCE NETWORK - FUNCTION

Shell eggs, as purchased by today's housewife, are rarely (Anon., 1982a), if ever, associated with salmonella food poisoning - salmonella accounting for approximately a third of all food poisoning cases (Silliker, 1982). Those eggs used for hatching, however, are considered to be an important (P. Wilding, pers comm) cause of the very high incidence of salmonellosis due to poultry (top implicated food; Anon., 1982a) in that they transfer Salmonella spp. (normally non-species specific) from breeding to growing stock.

At present, due to improvements in the management of poultry flocks, salmonellosis is not considered by the poultry industry to be of major economic importance (C. Baxter-Jones, pers comm), rather it is the public health aspect of salmonellosis which continues to cause concern. Although the acute enteritis (1 - 4 days duration) resulting from a salmonella infection is relatively harmless in the healthy adult, rarely causing mortality, this is not so for the compromised host eg. children under five, elderly people (over 65) and patients subject to medication or post-operative care (Knothe, 1984). Salmonella outbreaks in these groups often occur in institutions and may result in several deaths - giving rise to widespread publicity. For instance, of the 24 patients who died in England and Wales during 1981 as a direct result of salmonella food poisoning 16 were aged 65 or over (Anon., 1982a); three of the 50 cases of salmonella poisoning at Ottershaw mental hospital died (Anon., 1982b). This is the

exception however. It is generally accepted that only a small fraction (possibly as few as 0.0001% in the USA - Silliker, 1982) of human salmonella cases are reported. An aspect that is often ignored when considering salmonellosis is the financial cost (in terms of lost working days, medical care, etc) to a country's economy. Canadian health officials reported (Anon., 1978) on the cost of two outbreaks. The first involved a family of six, of which five members were infected - the "cost to the community" was estimated to have been in excess of $4,000; the second case (caused by the use of "Grade Crack" eggs in the ingredients of cake batter and icing) involved 44 cases and was estimated to have cost over $23,500. Human salmonellosis is still therefore a significant health problem, even if this fact is not recognised or is ignored by the poultry industry. Improvements in food handling practices could greatly reduce the number of salmonella outbreaks (Knothe, 1984) but there is a need to eradicate the organism in the foods most commonly associated with the disease. In poultry this could be carried out most successfully on the production farm, by breaking the cycle at its weakest point - the hatching egg (C. Baxter-Jones, pers. comm.) - all other treatments such as competitive exclusion (discussed on pp 247) should be seen only as adjuncts to this fundamental principle.

The major contaminants of the surface of eggshells (average 9.5 - 3,100 x 10^3 org./shell) are Gram-positive bacteria derived from dust, soil and faeces (Haines, 1939; Board et al., 1964). The majority of organisms that cause rotting of eggs are presumed to be extra-genital in origin. Indeed, less than 1% of nest clean eggs rot during prolonged storage (Brooks and Taylor, 1955). Thus some agency is required for the translocation of rot producing

bacteria across the shell (Board, 1966). The limited and mainly circumstantial evidence supports the view also that Salmonella in hatching eggs occur on the shell surface (Baker et al., 1980) and, as fumigation does not ensure control of salmonella transmission, it is assumed (C. Baxter-Jones, pers. comm.) that these organisms probably penetrate the shell prior to fumigation. Indeed, current studies are concerned with the use of disinfectants and flooding of the pore canals in attempts to kill Salmonella. The size of the hatching operation, in which many thousands of eggs are set at any one time, is another factor in the effective control of Salmonella. Thus a typical isolation rate for salmonellas in a commercial hatchery of 1 in 10,000 eggs (C. Baxter-Jones, pers. comm.) is significant when upwards of 50 - 100,000 eggs are present in the incubator. Indeed, the magnitude of the problem can be appreciated if the vertical transmission pattern is studied using the Binomial Distribution as has been done (Edson, 1980) in order to predict the probability of eliminating a pathogen, Mycoplasma meleagridis, from turkey hatching eggs. It has been computed that even low transmission rates of salmonellae (6×10^{-5} for a hatch of 50,000 or 6×10^{-4} for a hatch of 5,000) may result in most of the poults being infected on hatching or shortly thereafter (P. Wilding, pers. comm.). Should this occur, then competitive inhibition by the Nurmi method (discussed on pp 247) would be negated.

Although bacterial penetration of the egg's integument has been studied repeatedly there was, until recently, a poor understanding of the relative contributions made by the various components of the egg integument to the egg's defence. Romanoff (1931) suggested that the cuticle played an important role in water proofing the underlying pores, a suggestion that was

indirectly supported by Haines and Moran (1940) who noted that rubbing the shell with sandpaper increased the incidence of rotting. Walden et al. (1956), Kraft et al. (1958) and Brown et al. (1965), who used a similar technique to that of Haines and Moran, concluded that the "porosity" of the shell determined water uptake and hence bacterial infection. Haines and Moran (1940) also observed that the translocation of water across the shell integument was an energy demanding process - the energy being expended in overcoming the various resistances of the integument. Energy was supplied in the form of heat, warm eggs being placed in a cold bacterial suspension. A pressure gradient (resulting from the contraction of the egg contents) causes water containing bacteria to be drawn into the pore canals. It was assumed that contamination of the underlying shell membranes occurred also. This was confirmed by Brant and Starr (1962). They showed also that the incidence of infection of a group of eggs was associated with the temperature differential between the warm egg and cold water. The highest incidence of rotting being associated with the greatest temperature differential. With heavy contamination (10^4 - 10^8 org ml^{-1} of water) appreciable infection (up to 30%) occurred even when the bacterial suspension was warmer than the eggs.

Another source of energy was proposed by Board (1966) who suggested that the osmotic potential of the albumen might be sufficient to promote the inward movement of fluid in a flooded pore canal. Board and Halls (1973a) examined this hypothesis but found that although eggs with a section of shell removed would take up water by osmosis there was no apparent osmotic effect operating in eggs with intact shells. Presumably this was because the osmotic potential of the albumen could only operate on a pore

canal that was already flooded - it could not effect flooding *per se*. It is feasible however that the albumen could promote water uptake following the initial flooding of the pore canal by other means.

Having established the role of the cuticle in water-proofing the pore canals and the need for energy to be expended in order to force water across the shell integument, Board and Halls (1973a) used cuticle-less eggs in an unsuccessful attempt to correlate water uptake with pore number. They suggested that degassing the dip water would lead to a more even uptake of fluid and hence a significant correlation. During the present study therefore water was treated in a variety of ways in order to try and correlate water uptake to pore number, functional pore area and water vapour conductance. It was hoped that these studies would identify the major determinants of fluid uptake once the cuticle has been breached.

It is evident from the above that studies of the passage of bacteria from the outer surface to the albumen of eggs must pay attention to the potential resistances (Fig. 1) imposed by the egg's integument - the cuticle, calcitic shell, shell membranes and limiting membrane. This has rarely been done to date. Moreover, as this integument contributes in many ways to the well being of the embryo, studies of bacterial infection of the albumen ought not to be divorced from the two most important attributes of the integument, water conservation and gaseous diffusion. As will be evident in the following review both functions provide a "biological perspective" for the examination of bacterial penetration of the integument.

Much of the literature on bacterial invasion of the egg's

integument emphasises the importance of shell porosity. As was noted by Board and Halls (1973a) this attribute has been determined in many ways viz staining of the pores in the shell (Rizzo, 1899), release of gas by eggs immersed in water under vacuum (Bryant and Sharp, 1934), permeability of shell to gases, liquids and particles (eg Haines and Moran, 1940; Alls et al., 1963, 1964) and evaporative weight loss in stored eggs (Tyler, 1945). A refinement of the last mentioned method, based on water vapour conductance, has been adopted by avian physiologists in their studies of embryo respiration in incubating eggs (Wangensteen and Rahn, 1970/1971). Moreover the cuticle (Board and Halls, 1973a) or the shell membranes (Paganelli et al., 1978) of hens' eggs do not appear to influence this modern method of determining porosity which provides a measure of a fundamental property of the shell.

Water vapour conductance

The water vapour conductance studies of Ar et al. (1974) were based on daily measurements of the rate of weight loss of eggs under known conditions of temperature, humidity and barometric pressure. This was achieved by placing the eggs in a vented desiccator over dry KOH pellets which maintained the humidity around zero. The authors proposed two equations to express functional shell porosity. One expresses the rate of water loss from an egg as "water vapour permeability" (K_{H_2O}):

$$\dot{V}_{H_2O} = K_{H_2O} \cdot A \cdot \Delta P_{H_2O}$$

where

$\dot{V}_{H_2O}$ = diffusive rate of water loss (cm^3 STP.sec^{-1})

K_{H_2O} = permeability constant of the shell (cm^3 STP.cm^{-2}.$torr^{-1}$.sec^{-1})

A = surface area of the shell (cm^2)

ΔP_{H_2O} = water vapour pressure difference across the shell (torr)

the other as "water vapour conductance" (G_{H_2O}):

$$G_{H_2O} = \frac{\dot{M}_{H_2O}}{\Delta P_{H_2O}}$$

where

G_{H_2O} = water vapour conductance ($mg.day^{-1}.torr^{-1}$)

$\dot{M}_{H_2O}$ = the rate of weight loss ($mg.day^{-1}$)

ΔP_{H_2O} = water vapour pressure differential across the shell (torr)

The second equation has the advantage that the volume of water vapour is expressed as a mass unit and does not require the measurement of the shell's surface area. The equation which applies up and until the shell is "pipped" and is a mathematical expression of the number and size of the pores in the eggshell, is an attribute that is fixed at oviposition.

Until recently water vapour conductance was thought (Board and Halls, 1973a) to be independent of an egg's cuticle or cover. Colacino et al. (1985) observed, however, that if the cover is thick enough, then its removal will increase water vapour conductance values.

Water vapour conductance is determined therefore by the effective pore area, shell thickness, time, temperature and the steepness of the diffusion gradient across the shell. Water vapour conductance values are probably crucial to the development of the embryo. Drent (1975) deduced that for an optimum hatch eggs need to lose approximately 16% of the water present at oviposition. As most of the energy needed for embryonic development is derived from the fat stores of the yolk the mass of metabolic water produced is only slightly less than the mass of fat utilised - in essence about 11 litres of water vapour must pass across the shell integument

over the 21 day incubation period required for the chicken egg (Rahn et al., 1979). As water is lost at a steady rate so it is replaced by atmospheric air. The resulting air cell is essential for the onset of pulmonary respiration which begins on approximately day 19 of incubation - extra oxygen being required for the strenuous hatching process.

Calculating water vapour conductance as G_{H_2O} allows the functional pore area, which may be more relevant than pore number alone, to be measured quickly and accurately without damage to the shell. Using this technique Rahn et al. (1981a) studied changes in porosity during the laying cycle of turkey hens. Conductance was found to increase by 17% from the beginning to the end of the laying cycle. This was due solely to an increase in pore number (determined by acid etching - pp 125), the effective pore radius remaining constant throughout. Similar results were obtained by Tullett and Smith (1983) who studied domestic ducks. Pore number was also found (Rahn et al., 1981b) to be directly affected by barometric pressure, the eggs of White Leghorn hens taking only two months to adapt and compensate for a barometric pressure increase from 480 torr to 657 torr.

As the water vapour conductance (G_{H_2O}) is an index of arguably the shell's major biological contribution to the wellbeing of an embryo, it was adopted in the present studies of shell porosity and water uptake, the assumption being made that water is essential for bacterial penetration of the shell.

CUTICLE

Research into the antibiotic treatment of incubating eggs to

control mycoplasma infection in poultry flocks provided additional information about the cuticle's role in water-proofing of eggs and the need for work to be done. Thus Alls et al. (1963, 1964) and Ekperigin and McCapes (1977), who immersed warm eggs in chilled solutions of antibiotics ca. 24h after oviposition, found that antibiotic uptake was enhanced if the cuticle was removed (0.2N HCl for 1 min) or the eggs were naturally cuticle-less. The observations discussed above led Board and Halls (1973a, b) to conclude that two attributes of the integument of eggs need to be recognised: (a) water repellency and (b) water resistance. The former, which is a problem of little commercial importance unless the water is heavily contaminated, can be overcome by reducing the water's surface tension (Board and Halls, 1973a). The latter, which these authors associated with the cuticle, is overcome when work is done by the methods noted above.

There are vague reports (eg Zagaevsky and Lutikova, 1944) which suggest that the cuticle is susceptible to bacterial penetration immediately following oviposition. My studies confirm this susceptibility - the cuticle undergoing a rapid maturation process following oviposition which significantly increases the cuticle's ability to resist bacterial penetration. The observation by Smeltzer et al. (1979), who noted a significant difference between the incidence of infection of the membranes underlying the shells of nest (10.5%) and floor eggs (15.3%), may be due to this feature also.

Ball et al. (1975), who assumed that the intensity of staining was associated with cuticle density, used the food dye Edicol Supra Pea Green H - as described by Board and Halls (1973a) - in studies of the cuticle of stored eggs. They found that the

cuticle was not affected by the age of the hen or by spraying with chlorine dioxide, but it was adversely affected by fumigation (formaldehyde), uric acid (from faeces) and brushes in egg washing machines. It was noted also that cuticle quality (determined by scoring the stained cuticle - dark colours being indicative of a good quality cuticle) deteriorated as an egg aged especially with storage at high temperatures. Little change occurred with eggs held at 5°C for 36 days compared with a definite decrease in stain intensity with those stored at 24°C. Although Ball and his co-workers noted changes in staining characteristics they did not associate such changes with alterations in the microstructure of the cuticle. This area has therefore been the subject of further study.

Once the cuticle has been breached with contaminated water the pore canals are vulnerable to infection. Indeed before the water resistance of the cuticle was recognised, the literature emphasised the role of "porosity" in the antimicrobial defence of eggs. The literature failed to draw a distinction between the number of pores, their size and geometry. Indeed from a review of the literature, Board and Halls (1973a) concluded that "porosity" was a nebulous term of no immediate practical significance. Emphasis was placed in this study, therefore, on examining the microstructure of the hens' egg cuticle and relating this to its ability to influence water vapour conductance, water uptake and bacterial penetration of the shell integument. These features determine cuticle "quality" - a nebulous factor that cannot be measured quantitatively. At present the best measure of cuticle "quality" - cuticles fitness to protect the developing embryo - is obtained by staining the cuticle, and indeed this method has

been used in several studies concerned with the measurement of cuticle quality, although the specific cause of the differing shades was not appreciated. An attempt was made therefore to relate staining characteristics to the cuticle microstructure.

SHELL AND LIMITING MEMBRANES

Should the cuticle's resistance be negated and contaminated water flood the pore canal, the shell membranes may prevent detectable bacterial contamination of the albumen for up to 20 days (Stokes et al., 1956; Fromm and Monroe, 1960; Garibaldi and Bayne, 1960). Although the subject of several studies (Board, 1968) the precise mechanisms involved are still not fully understood. However it has been postulated that: the mesh-like physical structure of the membranes is restrictive (Haines and Moran, 1940), the membranes possess a chemical defence system (Stuart and McNally, 1943; Korotkova, 1957), or the antimicrobial factors in the albumen make the membranes an unfavourable environment for bacterial growth.

It was suggested (Kutchai and Steen, 1971; Lomholt, 1976a; Tullett and Board, 1976 and Kayar et al., 1981) that the shell membranes may also offer a variable resistance to gaseous conductance - the sudden decrease in the shell integument's resistance to gaseous conductance that is a feature of the incubated fertile hens egg being attributed to changes associated with the membranes.

The membranes appear to be capable therefore of restricting the passage of bacteria and gases. Studies in either of these fields has been hindered by the lack of fundamental knowledge concerning the structure, and to a lesser extent the chemistry, of the shell membranes. This was one reason why, rather than studying bacterial

penetration of the membranes in isolation, an integrated approach was adopted in this study. The electron microscope was used to follow the structural development of the membrane from the magnum through to the changes brought about in the oviposited egg by incubation or bacterial penetration. The EM observations were complemented by a study of the gaseous permeability of the shell integument, and finally bacterial penetration of the shell membranes. Where possible EM observations should always be supported by other techniques - EM observations being open to the valid criticism that what is being observed is an artefact of preparation.

Shell membranes - oxygen permeability

The avian embryo develops within the rigid calcitic shell of the cleidoic (closed box) egg in which, except for oxygen, all the materials required for embryo development and the initial maintenance of the chick post hatching are present. Oxygen is obtained and carbon dioxide and water are lost by diffusion, a process that does not require any metabolic energy expenditure by the chick (Rahn et al., 1979). During the 21 day incubation period the average chicken egg will take up about six litres of oxygen and give off 4.5 litres of carbon dioxide and 11 litres of water vapour (Rahn et al., 1979). The oxygen diffuses through the pore canal (the number and configuration of which determine the shell's permeability), across the shell membranes to the chorio-allantoic membrane - the embryo's gas exchange organ.

Wangensteen et al. (1970/1971) reported that the potential of the shell and outer membrane of chicken eggs to allow oxygen diffusion did not change during incubation. Kutchai and Steen (1971) on the other hand reported a change in potential, the

shells of eggs early in incubation having a lower conductance compared with those late in incubation. This was confirmed by Lomholt (1976a) and Tullett and Board (1976). Thus it was found that the oxygen flux across the shell integument increased ten-fold approximately 22% of the way through incubation (Tullett and Board, 1976). This change enables the oxygen permeability potential of the egg integument to accomodate the maximum demands of the chick immediately before pipping and hatching (Rahn et al., 1974). These early workers tended to invoke arguments about the influence of the water content of membranes, especially the inner one, on oxygen movement.

Wangensteen et al. (1970/1971) showed that water caused the membrane fibres to expand, thereby leading to a decrease in the membranes permeability. They contended, however, that this effect would not be sufficient for the change noted in the resistance to gas flux. Kutchai and Steen (1971) found that the water content of shell membranes of incubated/fertile eggs decreased from 70% to 40% during 17 days of incubation. As artificial drying caused an increase in the permeability of the membranes to oxygen/carbon dioxide, they concluded that water loss, possibly associated with an increased colloidal osmotic effect from the albumen, caused this increase in permeability. Indeed they postulated that a layer of water in the interstitial spaces of the membrane thins as the membranes dry and its eventual rupture creates air channels. Rahn et al. (1974) noted however that under conditions of constant temperature/humidity the loss of water vapour from the egg remains "remarkably constant" throughout incubation. In a more recent study Kayar et al. (1981) also argued that the loss of the inner membrane's resistance was associated with its water content which

apparently declined as the resistance of the inner membrane fell from 88 to 12% of the total offered by the whole.

The development of the chorio-allantoic membrane was studied in conjunction with oxygen uptake by Ackerman and Rahn (1980). They found that the rapid increase in oxygen uptake between days six and nine was paralleled by chorio-allantoic membrane development (measured in terms of % area of shell covered). They contended that the oxygen flux was confined to those areas of the shell covered by the chorio-allantoic membrane and that the oxygen permeability (K_{O_2}) of the shell (including the outer membrane) was constant. None of the studies considered any potential contribution by the limiting membrane.

Shell membranes - delay in bacterial penetration

The means by which bacteria penetrate the membranes is still not fully understood (Tung et al., 1979). Once contamination of the outer surface of the membranes has occurred, a lag of up to 20 days may precede demonstrable contamination of the albumen - unless the contaminants are supplied with iron (Stokes et al., 1956; Fromm and Monroe, 1960; Garibaldi and Bayne, 1960; see pp 94). The retention of microorganisms was attributed (Stokes et al., 1956) to the shell membranes providing a mechanical barrier to bacterial penetration.

Further circumstantial evidence supporting the view that the membranes impede bacterial penetration comes from the work of Board and Board (1967), Smeltzer et al. (1979) and Wilding and Baxter-Jones (1982). They challenged eggs with microorganisms and then replaced the yolk and white with a nutrient agar containing a tetrazolium salt. The tetrazolium salt was reduced

by the organisms which penetrated the membranes and the insoluble purple dye, formazan, was formed. For this method to work, it is necessary to postulate that some of the organisms that penetrate the shell are restrained by the membranes. If they were not, then none would be present to grow and reduce the tetrazolium salt. All these workers noted the growth of bacteria in the shell membranes and concluded that these structures do impede, at least temporarily, bacterial movement. Likewise the studies with whole eggs by Board and Ayres (1965) showed that pseudomonads applied to the outer surface of the membranes (and held at 10°C) grew and were contained within the membranes for upwards of seven days. It is notable that only Candlish (1972) and Tung et al. (1979) - who failed to find any evidence of bacteria accumulating against or digesting the limiting membrane - have reported on the role of the limiting membrane in delaying bacterial penetration of the egg contents.

It appears from the only study to date (Seviour and Board, 1972) that once the membranes have been infected with a heterogeneous population of microorganisms the conditions in the membranes select Gram-negative organisms in preference to the dominant (Gram-positive) contaminants of the shell surface (Board et al., 1964). Temperature was found to be an important selective agent (Seviour and Board, 1972). Thus in eggs stored at 37°C the coliform organisms achieved dominance whereas at 30°C or less the pseudomonads did so. The Gram-negative organisms did not maintain their dominance at 37°C however, micrococci becoming dominant by the 21st day of incubation. Seviour and Board (1972) surmised that these organisms had been selected when the a_w fell due to evaporative water loss.

After the primary phase of bacterial multiplication in the

shell membranes there is a decline in numbers and then a secondary phase of multiplication which leads to addling of the egg. Brooks (1960) attributed this to a spontaneous change in the shell membranes. Other workers (Board, 1964; Board and Ayres, 1965) found that renewed growth only occurred when the yolk made contact with the inner shell membrane of eggs held in the range 10 - 30°C. The duration of the lag between primary and secondary multiplication reflects the rate at which the yolk travelled towards the membranes. Thus it is effectively a function of the storage temperature, there being a long lag with low temperatures and _vice versa_. In eggs stored at temperatures of 37°C or above or 10°C and below there is only one phase of multiplication (Board and Ayres, 1965). This has been attributed to the initiation of rots by contamination in the albumen that collide with the yolk. Bacterial multiplication within the membranes is also determined in part by the magnitude of the bacterial challenge, a large innoculum (10^5 cfu/egg) applied directly to the inner membrane penetrating rapidly, especially if the eggs are stored at 37°C (Hartung and Stadelman, 1963).

Bacterial penetration - physical barrier

The ability of the membranes to delay bacterial growth was studied by Haines and Moran (1940) who replaced the egg contents with a bacterial suspension and then applied suction to the outside of the shell. Fluid drawn through the shell and membranes did not contain bacteria whereas that drawn through shells lacking membranes did so. Whilst these results have been confirmed by others (Walden _et al._, 1956; Garibaldi and Stokes, 1958), the conclusion that the membranes act as bacterial filters is open to criticism simply because the bacteria were made to traverse the shell in a direction

opposite to that by which contamination normally occurs. In attempts to simulate the latter Bean and McLaury (1959) and Williams and Whittemore (1967) stood the shell (with or without one or both of its membranes) in a suspension of bacteria and attempted to recover organisms from broth contained within it. They found that, although the shell plus membranes offered greater resistance to bacterial movement than did the shell alone, there was little evidence to support the contention that the shell and membranes played a major role in preventing bacterial penetration.

In a study of changes in the fine structure of shell membranes contaminated with Pseudomonas aeruginosa, Brown et al. (1965) reported zones of clearing in the "interstitial 'albuminous' matrix material" - material present between the fibres - which they attributed to proteolytic breakdown brought about by microorganisms. Hartung and Stadelman (1963) found a significant relationship between age of egg and degree of bacterial challenge, the older the egg the more rapidly bacteria penetrated the shell membranes. These authors attributed this to the alkaline hydrolysis of what they described as "protein" in the interstices of the shell membrane fibres. It was suggested that this hydrolysis was caused by the rapid increase in pH of the ageing albumen (Sharp and Powell, 1931).

Brown et al. (1965) also studied the permeability (using haemoglobin, 1% soluble starch and 10% hens' egg albumen) of the inner membrane before and after bacterial penetration. Their results led them to postulate that enzymes were "actively involved in the penetration process" even though they found no evidence that the membrane fibres per se were digested or degraded in any way. In view of my studies on changes in the membranes in the oviduct,

it is highly unlikely that much material was present in the interstices of the membranes and some other explanation is called for. It should be noted that the studies of Garibaldi and Stokes (1958), Board (1965a), Wedral (1971) and Tung et al. (1979) provided no evidence for digestion of the membrane by bacterial proteases. Indeed Wedral (1971) tested commercially prepared lipase, phospholipase C, protease, proteinase, neuroaminidase, collagenase, acid and alkaline phosphatase, elastase, pepsin, trypsin, hyaluronidase and a combination of protease and lipase but none of these aided bacterial penetration of the membranes. The extra, ecto and intracellular enzymes from Salmonella typhimurium LT2 and Pseudomonas aeruginosa were equally unsuccessful.

Wedral (1971) suggested that the shell membrane's impediment of bacterial penetration might well be associated with the formers attraction of and perhaps subsequent adhesion to bacteria through a charge effect, such a mechanism being dependant on the charges in the membranes and bacterial cell surfaces. She contended that, once the bacteria had adhered to the shell membranes, they would grow and excrete chelating agents thereby modifying the membrane's properties such that the movement of bacteria was aided. Also the chelatation of metal ions would increase bacterial growth (see pp 106). In practice she was supporting a contention of Board (1966), namely that through trapping bacteria and thereby protecting them from the albumen, the shell membranes may well increase the eggs vulnerability to the infection.

Bacterial penetration - chemical defence

Recently Doyle (1984) used a novel technique to study egg infection with Campylobacter jejuni. He cemented a cylinder of

faeces contaminated with C. jejuni to the outside of the eggshell and held eggs so treated at a range of temperatures for known times. The eggs were then assayed for the degree of penetration which was assessed in three ways: by sampling the egg contents, aseptically removing the inner and outer shell membranes and adding them to enrichment medium or swabbing the inner surface of the shell below the base of the cylinder. Doyle isolated the organisms from the inner shell surface and from the shell membranes but not from the egg contents. As nothing is known about the fate of such an oxygen-sensitive organism in the harsh environment offered by albumen (pp 98), Doyle's observations may reflect the contribution of the albumen to an egg's chemical defence rather than the physical defence of the membranes.

Brooks and Taylor (1955) postulated that the shell membrane per se contained antimicrobial substances. Subsequent studies have shown however that intact (Board, 1965b) or comminuted (Stokes and Osbourne, 1956; Garibaldi and Stokes, 1958) membranes allow good growth of the common contaminants of eggs when suspended in a non-toxic solution of mineral salts. The available evidence led Board (1966) to suggest that the lag period prior to demonstrable contamination of the albumen is due to the antimicrobial nature of the underlying albumen (pp 98) rather then the restrictive nature of the membranes.

Garibaldi and Bayne (1962a, b) noted that minute traces of iron (4.8 ppm) in wash water increased the incidence of rots in stored eggs. Board *et al.* (1968) found that when iron is introduced into the egg via wash water it becomes localised in the shell membranes where it stimulates bacterial growth. The latter authors also found that $FeSO_4$ behaved in a similar manner (adhering to the membranes) when they included it in an inoculum on the inner

membrane in the air cell. This apparent sequestration of Fe^{3+} by the shell membranes was investigated in the present study. It appears therefore that the ovotransferrin of the albumen through denying iron to the bacteria (Board and Hornsey, 1978) restricts bacterial multiplication in the membranes. Although there is no evidence that ovotransferrin occurs in the shell membranes, the potential of ovotransferrin to influence an environment even though physically isolated from it was demonstrated by Feeney and Nagy (1952). Garibaldi and Bayne (1962b) contended that for growth to occur in the membranes the ovotransferrin need not be completely saturated, rather the iron acts by providing a sufficient quantity of this essential trace nutrient at the shell membrane for the growth of the organism, resulting in contamination of the albumen.

As noted above the persistence of infection in the membranes may reflect a subtle interplay of their structural properties (ie. acting as filters) and an inimical environment imposed on them by the underlying albumen. It was for this reason that microbiological and EM techniques were combined in this study in an attempt to elucidate the interplay of the shell membrane's structure and the probable inimical environment they offer contaminants because of the properties of the underlying albumen.

ALBUMEN - BACTERIAL DEFENCE

The viscous, heterogeneous albumen of the hen's egg is laid down around the yolk in three distinct layers: an outer layer of thin albumen, a middle layer of thick albumen and an inner layer of thin albumen. The major difference between the thick and thin

albumen is their mucin content, the former containing about four times as much ovomucin. Their protein content is similar, the total protein content accounting for 10% of the albumen. The free carbohydrate, mainly glucose, amounts for 0.5% of the albumen and the lipid 0.02 - 0.03% although this has yet to be characterised. There are also minute quantities of inorganic elements in bound or dissociated form whose concentration is influenced by the hen's diet, environment, temperature and age of bird (Powrie, 1977).

Of the 50 recognisable proteins that have been isolated from albumen, 19 can be detected by starch-gel electrophoresis (Lush, 1961) but only five are present at a concentration of greater than 1% (ovalbumen - 54% of protein present, ovotransferrin - 12%, ovomucoid - 11%, ovomucin - 3.5% and lysozyme - 3.4%; Parkinson, 1966).

Non-antibacterial proteins

Ovalbumen

Ovalbumen, a phosphoglycoprotein (MW approximately 45,000), is the predominant protein (54%) in albumen. It has three components (A_1, A_2 and A_3) which differ in their phosphorous content (Powrie, 1977). Ovalbumen is the only protein (other than lactalbumen) known to contain all the nutritionally essential amino acids (Parkinson, 1966).

Ovomucin

Ovomucin is the glycoprotein responsible for the gel-like structure of thick albumen where it occurs as flexible microscopic fibres. Brooks and Hale (1959) suggested that the gel-like

structure resulted from ovomucin and lysozyme complexing into a crosslinked structure. The thick albumen surrounds the yolk in an "albuminous sac", ensuring that the yolk is held away from the shell membranes, which may be heavily contaminated prior to detectable contamination of the albumen. This aspect of bacterial contamination was investigated in this study. During storage the gel-like structure is gradually lost, causing a "thinning" of the egg albumen which allows the yolk to float up and make contact with the shell membranes. In the incubated fertile egg albumen thinning might be beneficial, allowing the albumen to pass more readily into the amnion, from where it can be utilized by the embryo. Conflicting proposals have been put forward to account for the thinning of egg white, eg; the thinning is caused by a reduction in the amount of naturally occurring ovomucin-lysozyme complex at pH 9 - 9.5, or interaction increases as the pH rises, causing albumen thinning (Powrie, 1977). The loss of lysozyme activity in stored eggs is assumed to be evidence for the latter. Robinson and Monsey (1971; 1972a, b; 1975) demonstrated that hen's egg ovomucin consisted of two components; alpha-ovomucin and beta-ovomucin which contained 1 and 10% sialic acid (w/w) respectively. It was postulated, and (Kato et al., 1975) supported the view, that complexing was the result of electrostatic interaction between the negative charges of the terminal sialic acid in ovomucin and the positive charges of the lysl ε-amino acid groups in lysozyme. Robinson and Monsey (1975) demonstrated that with incubation at 37°C, thinning of the albumen was associated with degradation of beta-ovomucin; indeed this can be taken as evidence that the loss of the gel structure was caused by a breakdown in the ovomucin-lysozyme complex. In a recent study Hayakawa et al. (1983) postulated that beta-ovomucin

influenced the solubility of alpha-ovomucin. Their results indicate that beta-ovomucin inhibits the formation of an insoluble alpha-ovomucin complex with lysozyme in neutral or slightly alkaline pH regions, ie the pH of fresh egg albumen. They also found that bound beta-ovomucin inhibits the aggregation of the alpha-ovomucin-lysozyme complex in thick egg white which results in the formation of a gel-structure.

By increasing the viscosity of the albuminous sac ovomucin helps impede motile bacteria (Gillespie and Scott, 1950). Ovomucin also inhibits viral haemogglutination (Parkinson, 1966).

Antibacterial proteins

The antibacterial nature of egg albumen has been appreciated for many years and was alluded to by Shakespeare in the tragedy King Lear. The first notable scientific study was carried out by Wurtz (1890) who studied the death of the typhoid bacillus and pyogenic cocci in albumen. He was the first to conclude that the albumen protects the embryo's principal food reserve, the yolk, from bacterial contamination. Laschtschenko (1909) was the first to record the lysis of vegetative cells and spores of Bacillus spp. in egg white. As heating the albumen to 65 - 70°C for 30 min. prevented lysis, he assumed that a thermolabile enzyme was responsible. This was confirmed by Fleming (1922) who named the enzyme lysozyme.

Lysozyme

Lysozymes (muramidases - N-acetyl hexosaminidase EC 3.2.1.17) are widely distributed in nature (Jolle's, 1969), occurring in the body fluids of vertebrates, invertebrates (Chain and Anderson, 1983;

Mochizuki and Matsumiya, 1983) and some plants (Pilet and Bernasconi, 1984). Lysozymes have been isolated from the eggs of various animal species, birds in particular are endowed with this enzyme. (Sibley, 1960, 1970; Sibley and Ahlquist, 1972). Their contribution to the total egg white protein ranges from 3-4% in the galliformes and anseriformes (Feeney et al., 1960; Feeney and Allinson, 1969) to only trace amounts in penguins (Manwell and Baker, 1973).

Avian lysozymes have been divided serologically into two categories (Arnon et al., 1974; Ibrahini et al., 1979), chicken-type lysozyme (lysozyme$_c$) and goose-type lysozyme (lysozyme$_g$). The former is a basic protein (pI 10.7, Poole et al., 1984) of 129 amino acid residues (MW 14,000) arranged in a single polypeptide chain (Jolles et al., 1963; Canfield, 1963). Three domains of the molecule have been identified; 1 - 40 (domain I), 41 - 101 (domain II) and 102 - 129 (domain III). It is the interactions between domains I and III (Hiromitsu et al., 1984), the four disulphide bridges and the numerous hydrogen bonds (Brown, 1964) that stabilize the tertiary structure. Lysozyme$_g$ on the other hand is a slightly larger molecule (MW 19,500 - 21,500). The molecule consists of 180 amino acids joined by four half cysteine residues, two disulphide bridges and numerous hydrogen bonds (Tranter and Board, 1982a). In its action upon Micrococcus lysodeikticus (Canfield and McMurray, 1967), goose lysozyme is three times as active as an equimolar amount of hen egg lysozyme. The structure of the catalytic site also appears to differ (Rupley, 1966) although Canfield and McMurray (1967) considered it probable that goose and hen lysozyme act at the same site in cell walls.

Lysozyme is not bactericidal per se, rather it acts on the rigid eubacterial cell wall, exposing the underlying cell membrane

which ruptures unless the medium is isotonic (Weibull, 1953; Zinder and Arndt, 1956). Berger and Weiser (1957) showed that chitin, a linear polymer of β(1 - 4) linked units of N-acetylglucosamine (NAG), was lysed by chicken egg white. The cell wall of M. lysodeikticus - the organisms used extensively in early studies because of the ease (in the pH range 4 - 10; Chang and Carr, 1971) with which its cell wall is lysed - is formed in large part from a highly water-insoluble polymer, peptidoglycan. Peptidoglycan consists of equimolar amounts of NAG and N-acetyl muramic acid (NAM), β(1 - 4) linked and interconnected by peptide chains attached to the D-lactyl group of some 50% of the NAM units. It is these β(1 - 4) linkages that are attacked by lysozyme$_c$ (Berger and Weiser, 1957; Salton and Ghuysen, 1960). Lysozyme$_g$ cannot hydrolyse large polymers of NAG (Charlemagne and Jollès, 1967) and is unaffected by NAG inhibitors; it is specific for NAM residues whose lactyl group is peptide linked (Dianoux and Jollès, 1967).

Lysozyme$_c$ has been isolated from members of the Galliformes and Anseriformes eg chicken, quail, pheasant, guinea-hen, turkey, duck (Fukamizo et al., 1983; Smith-Gill et al., 1984) and from a member of the order Columbiformes (pigeon egg white; Gavilanes et al., 1982). Type-g lysozymes have been found in nine orders; Anseriformes, Struthioniformes, Rheiformes, Apterygiformes, Tinamiformes, Podicepediformes, Spheriisciformes, Casuriformes and Charadriiformes (Prager and Wilson, 1974). The egg white of the Black swan contains both types (Morgan and Arnheim, 1974). The absence of one lysozyme type in the egg white does not mean that it does not occur in the bird. For example both enzyme types occur in the chicken (Hindenburg et al., 1974) and duck

(Arnheim, 1974) although only lysozyme$_c$ occurs in the egg whites.

Only 5 - 10% of the cell wall of Gram-negative bacteria is in the form of peptidoglycan (Rogers and Perkins, 1968). This small amount is protected from lysozyme by laminae of lipoproteins and lipopolysaccharides which provide a barrier to diffusion. It is noteworthy therefore that Gram-negative bacteria are the dominant contaminants of rotten eggs (Board, 1964, 1965b). Some Gram-positive organisms are resistant to lysozyme due to cell wall modicications, eg teichoic acid, a highly negatively charged polymer present in the cell wall of Staphylococcus aureus and other bacteria, can bind basic proteins such as lysozyme and thereby confer resistance. Streptococcus pneumoniae is resistant to lysozyme digestion due to an unidentified "non-peptidoglycan" component of the cell binding the lysozyme molecule (Ohno et al., 1983). The results of a study by Mendelson et al. (1984) suggest that the cell wall polymers of Bacillus spp. can assume a range of structural states during helical growth. These determine the quantitative aspects of macrofibre shape and the sensitivity of the walls to attack by lysozyme. The absence of N-acetyl groups is sufficient to render Bacillus cereus resistant to lysozyme (Araki et al., 1972). A similar situation exists in the cell wall of B. anthracis where approximately 88% of the glucosamine residues and 34% of the muramic acid residues of the cell wall peptidoglycan contain unsubstituted amino groups (Zipperle et al., 1984).

Lysozyme will also combine with ovomucin to restrict microbial growth by increasing albumen viscosity (discussed on pp 261).

Although Fleming's (1922) observations on the enzymatic nature of the lysis of bacteria in albumen and his proposal of the name lysozyme led to the enzyme being accorded a cardinal role

in the antimicrobial defence of the egg, little if any supporting evidence has been published (Board and Fuller, 1974).

Ovotransferrin

The transferrins are a family of homologous proteins which have the ability to bind two ferric iron atoms (Aisen et al., 1966). They transport iron to the developing red cells for the synthesis of haemoglobin (Harris, 1983).

Ferric iron (Fe^{3+}) is extremely insoluble at physiological pH so that ferric hydroxide will tend to polymerise and precipitate once the Fe^{3+} concentration exceeds approximately 10^{-17}M (Spiro and Saltman, 1969).

In order to transport iron under these conditions, both vertebrates and bacteria have developed specific iron-transport compounds. The vertebrates transport iron as a complex with the glycoprotein transferrin (Wing-Ming and Azari, 1982). Transferrins (eg lactotransferrin) also occur in the secretions and milk of mammals (Wing-Ming and Azari, 1982).

The transferrins have been divided into three broad groups; those found in serum - transferrins (MacGillivray et al., 1983), those found in milk - lactotransferrins (Arnold et al., 1980), and those found in egg white - ovotransferrins (Williams et al., 1982). The nomenclature is confused however by the use in the literature of a number of other names eg serotransferrin, siderophilin and beta-1-metal combining protein refer to transferrin; lactoferrin and red protein refer to lactotransferrin and conalbumen to ovotransferrin.

The yolk, the prime source of nutrient for the embryo, contains the bulk (1mg/egg) of the egg's iron reserve (Halkett

et al., 1957). Ovotransferrin has been isolated from the yolk, and from the albumen - which is essentially the embyro's water reserve and only contains about 138µg of iron (Romanoff and Romanoff, 1967). Ovotransferrin accounts for approximately 4% and 13% respectively of the total protein in these two structures, (Marshall and Deutsch, 1951; Tranter and Board, 1982a). As yet the biological function of ovotransferrin in the hen's egg has not been identified.

Frelinger (1970) noted that the pigeon embryo absorbed a large amount of transferrin via yolk or albumen, or both. The transferred protein represented the major portion of the young squabs serum transferrin even though the squab starts to actively synthesise transferrin just prior to hatching. However the transferrins found in the pigeons blood, egg white, egg yolk, crop milk and, probably, semen, all have equal electrophoretic mobility (Frelinger, 1970) whereas those found in the hen differ slightly. The hen's serum transferrin molecule and ovotransferrin molecule differ in that the latter lacks sialic acid in the carbohydrate group (Williams, 1962). Williams (1962) and Ogden et al. (1962) contend that the transferrins are probably controlled by the same genetic locus. The differences between serum and egg transferrins are therefore minimal. More recent studies indicate that there are several polymorphs of the transferrin molecule present in hen serum, egg yolk and egg albumen. Rashid (1981) reported finding two transferrin types in hen serum, both having a similar amino acid construction but differing in the carbohydrate-terminal half of the molecule (a situation similar to that found between hen serum transferrin and ovotransferrin); no physiological difference existed between the two and they both donated iron

to chicken embryo red cells at an equal rate. Three forms of transferrin have been isolated from the hen's egg yolk (Kierek-Jaszczuk, 1981). They differ in the number of sialic residues (0 - 2) contained within the transferrin molecule. As was discussed above, Williams (1962) found that the only difference between the hen serum transferrin and ovotransferrin was the presence of sialic acid in the former. These studies tend to support the earlier reports of Lush (1961) and Ogden et al. (1962) who, using electrophoretic techniques, found three ovotransferrin types in chicken egg white - types A, B and AB. It is notable that these authors also reported corresponding electrophoretic differences in the serum transferrin of the particular hen strain under observation.

Is it possible, that in order to maintain rapid development, the chick embryo utilizes polymorphs of ovotransferrin (undetected due to their diversity) which originate in the egg, gradually replacing them with serum transferrin after hatching? Alternatively if the embryo no longer utilizes the transferrins found in the yolk/albumen, do the ovotransferrins represent the redundant remains of such a system? It is known that as a percentage of the total protein, serum transferrin decreases from approximately 8% in the 13 day old embryo to around 3% in male chicken serum (Marshall and Deutsch, 1951).

The ovotransferrin molecule is a prolate ellipsoid (68A by 28A, Yeh et al., 1979), of 705 amino acid residues (MW 77,770), including a 19 residue presequence (Williams et al., 1982). The single polypeptide chain can be cleaved - through a nine residue connecting peptide (Williams et al., 1982) - into two immunologically distinct domains (Wing-Ming et al., 1982; Brown-Mason and Woodworth, 1984). The domains differ in the histidine, alanine and methionine content

and the alpha-helical content of the NH_2-fragment backbone, which is only half that of the CO_2H-fragment. However each domain (NH_2- and CO_2H-) contains and iron binding terminal. Three histidines are involved at each iron-binding site, one histidine binding anions and one binding metal ions (Alsaadi et al., 1981). Anions are thought to be necessary as a binding ligand between the metal and protein, stabilising and protecting the bond (Williams and Woodworth, 1973). Ovotransferrins utilize either carbonate or bicarbonate to satisfy this requirement (Warner and Weber, 1953). It is the stringent association of metal binding properties with anion binding requirements that is the most characteristic feature of the transferrins and makes them unique in metallobiochemistry (Aisen and Listowsky, 1980). However the reported ability (Wing-Ming et al., 1982; Wing-Ming and Azari, 1982) of the separate domains to take up iron atoms and then bind to the red blood cell of a chicken embryo has been disputed by Brown-Mason and Woodworth (1984). Both groups agreed though that equal mixtures of the two groups show an immunochemically indistinguisable reaction from that of the intact molecule.

The stability of the ovotransferrin molecule is enhanced by metal ion-binding (Azari and Feeney, 1958; 1961). This may be due to metal chelation giving rise to new bonds and hence to a modified secondary or tertiary structure. Although ovotransferrin commonly complexes with iron it will complex with other metals. Tan and Woodworth (1969) used displacement studies to study the relative stabilities of ovotransferrin complexes and found the following order of stability;

$$Fe^{3+}\ Cr^{3+};Cu^{2+}\ Mn^{2+};Co^{2+};Cd^{2+}\ Zn^{2+}\ Ni^{2+}$$

the metal complexes are stable at alkaline pH's (9 - 10) but

will dissociate in acid solution (pH 6.5).

Fe^{2+} does not appear to be bound by ovotransferrin (Gaber and Aisen, 1970) although Bates et al. (1973) claimed that ovotransferrin would bind to Fe^{2+} and oxidise it with atmospheric oxygen using ferroxidase.

Iron normally binds in a random fashion to the ovotransferrin binding sites, unless a low molecular weight chelator is present such as nitrilotriacetic acid in which case once iron has bound to one site the "availability" of the second site appears to be diminished (Donovan et al., 1976). Similarly, the release of iron to chick red blood cells is random (Williams and Woodworth, 1973).

It was probably because avian egg albumen contains the highest concentration of transferrin to be found in nature (Clark et al., 1963), that ovotransferrin was the first of the transferrins to be separated and identified and identified as an individual protein (Osbourne and Campbell, 1900). It was over 40 years later that Schade and Caroline (1944) made the important observation that only iron could overcome the bactericidal effects of chicken egg albumen on organisms that were known not to be avidin/biotin sensitive. The practical implications of these findings were emphasised by the studies of Garibaldi and Bayne (1960, 1962a, b). They (1960) noted that iron salts had a "striking effect" on the rate and the extent of Pseudomonas spoilage in experimentally infected shell eggs. Other Gram-negative contaminants, including strains of Proteus, Paracolobactrum, Alcaligenes, Aerobacter, Achromobacter and Salmonella were found (Garibaldi and Bayne, 1962a) to be similarly affected. The need to reduce to a minimum the iron content of water used to wash shell eggs also became apparent: on one farm Garibaldi and Bayne (1962b) found that raising the iron concentration

of the wash water from 0.4 to 10ppm increased the Pseudomonas spoilage from 0.8% to 2.5% after 48 days storage at 13°C. On a second farm the authors substituted "contaminated" (4.8ppm) water for water with a low (0.2ppm) iron level - Pseudomonas spoilage of stored eggs increased from 0.8% to 6.2%.

Although ovotransferrin only causes stasis, this effect is accentuated by the alkaline pH (9 - 9.5) of the stored egg (Feeney and Nagy, 1952; Seviour and Board, 1972; Board and Halls, 1973c; Khoury-Doughly and Spencer, 1976; Tranter and Board, 1984). If ovotransferrin is saturated then bacterial growth can occur (Baltimore et al., 1982). It is not the presence of ovotransferrin per se that inhibits growth, but its potential to complex with free iron.

Bacteria overcome ovotransferrin-inhibition provided they have an efficient iron-scavenging/transport system ie siderophores that operate effectively in a medium. There are two classes of siderophores; catechols (phenolates) which are commonly found in bacteria and the hydroxamates commonly found in fungi. Most members of the Enterobacteriaceae possess a prototype of the catechol siderophore, in the form of enterobactin (Pollack and Neilands, 1970) or enterochelin (Cox et al., 1970). Tranter and Board (1982) noted however that the hydroxyl groups of enterobactin are vulnerable to oxidation (and the lactone to hydrolysis) at the alkaline pH found in stored egg albumen (pH 9.4). The authors contended that there "is too little evidence in support of the claims that microorganisms can counter the Fe^{3+}-deficient state.... ...through the production of iron scavenging compounds.....", accounting in part, for ovotransferrins position as the egg's major chemical defence.

Ovomucoid

Ovomucoid is a non-heat coaguable glycoprotein - MW 28,000 (Parkinson, 1966), which is able to inhibit trypsin (Lineweaver and Murray, 1947).

The four types of ovomucoid isolated from avian albumen by Rhodes et al. (1960) differed in their sialic acid content and the type of trypsin with which they would complex: one primarily inhibited trypsin, another inhibited chymotrypsin, a third inhibited equal molar amounts of trypsin and chymotrypsin and the fourth type inhibited twice as much trypsin as chymotrypsin, separately or simultaneously. A later study by Feeney et al. (1963) revealed that chicken ovomucoid inhibited trypsin only, the reported weak inhibition of chymotrypsin being due, they surmised, to contamination with ovoinhibitor. Ovomucoid's antitryptic activity is lost more rapidly at pH 9 than at acid pH, retaining only six percent of its activity after 30 minutes at 80°C in 9M urea (Stephens and Feeney, 1963). The egg's ovomucoid is utilised by the developing embryo, passing from the albumen to the amnion where it is transported via the gut to the yolk sac to be catabolized by proteases and glucosidases (Oegana and Jourdian, 1974a, b). By day 12 ovomucoid may be detected in the embryo's serum (Board and Hornsey, 1978).

Ovoinhibitor

Ovoinhibitor is a glycoprotein with a molecular weight of approximately 46,500 (Tomimatsu et al., 1966), and accounts for about 0.1% of the albumen protein. Although superficially chicken ovoinhibitor has many physical, chemical and biological properties similar to chicken ovomucoid (eg ability to inhibit trypsin) there

are also marked differences, viz molecular weight, enzyme-inhibitory specificity and carbohydrate content. Moreover there is considerably less carbohydrate in ovoinhibitor (Tomimatsu et al., 1966).

Ovoinhibitor was first isolated by Matsushima (1958) who found that it inhibited proteases from Bacillus subtilis and Aspergillus spp.; subsequent studies revealed that ovoinhibitor also inhibited trypsin (Feeney et al., 1963), turkey trypsin and chicken chymotrypsin (Ryan et al., 1965). Ovoinhibitor is a "double-headed" inhibitor (Rhodes et al., 1960), one mole of inhibitor combining simultaneously with two moles of trypsin and two moles of chymotrypsin (Tomimatsu et al., 1966). The latter worker studied the reactions of mixed proteolytic enzymes. Their findings confirmed those of Feeney et al. (1960; 1963) that: a) the inhibition of one enzyme (trypsin or chymotrypsin) was essentially unaffected by the presence of the other, suggesting separate binding sites; and b) similar results were obtained for mixtures of trypsin and subtilisin although in mixtures of chymotrypsin and subtilisin the inhibition of one enzyme was definitely affected by the presence of the other, suggesting that those two enzymes compete for the same or adjacent sites.

Ovoinhibitor is more stable under acid conditions, retaining about 95% of its activity for 15 minutes at pH 3 - 5; it loses its activity in less than 15 minutes at pH 7 - 9 (Tomimatsu et al., 1966).

Board and Hornsey (1978) noted that whilst Ayres and Taylor (1956) had implied that ovomucoid (and by implication, ovoinhibitor) played an antimicrobial role in the egg, there was "no direct evidence" to support this. Board and Fuller (1974) suggested that ovomucoid and ovoinhibitor may inhibit proteases that are

released by cells which lyse within the egg eg those spermatozoa that do not participate in fertilisation. They also observed that bovine colostrum inhibitor may protect immunoglobins from digestion in the alimentary tract and suggested that the inhibitor in egg white might play a similar role.

Ficin/papain inhibitor

Fossum and Whitaker (1968) isolated a previously unidentified inhibitor from egg white. The protein reacted (1:1) with either papain of ficin (plant proteinases), the enzymes competing with each other for the binding site (Fossum and Whitaker, 1968). The purified protein had a molecular weight of about 12,700 and lacked a carbohydrate moiety - a common feature of many other low molecular weight inhibitors (Laskowski and Laskowski, 1954). The authors found that the molecule was virtually unaffected by pH, although it was slightly less stable at pH 9 than at pH 4. The absence of a pH effect indicates that the forces holding the complex together are mainly hydrophobic. There is no proposed role for this inhibitor in the egg's antimicrobial system.

Ovoflavoprotein

The apoprotein of ovoflavoprotein is a glycoprotein with a molecular weight of 32,000 (Farrell et al., 1969). Riboflavin forms the flavin moiety in flavoprotein, all the riboflavin molecules present in the egg being bound to the apoprotein molecules on a one to one basis (Rhodes et al., 1958).

Riboflavin is adsorbed from the digestive tract into the blood stream where it combines with the apoprotein. The flavoprotein is then transported to the developing yolk where

it is stored ready to be utilised by the embryo (White et al., 1976). Although the yolk apoprotein is derived from the hen's serum, the albumen apoprotein is a product of de novo synthesis in the oviduct. The latter flavoprotein is responsible for the greenish tinge associated with hen's egg albumen. It was the lack of this tinge that first led investigators to suspect riboflavin deficiency in a strain of hens suffering from total embryo mortality (Buss, 1969). Buss (1969) found that through a genetic defect the hens lacked the serum apoprotein and consequently the riboflavin was being excreted in the urine rather than passing into the egg. This demonstrates the physiological importance of flavoprotein. Although they originate from different sources the flavoproteins in the serum of the hen (Winter et al., 1967a, b), the egg albumen and the egg yolk (Ostrowski et al., 1962, 1968) are serologically identical (Farrell et al., 1970), hence Clagett's (1971) suggestion that the riboflavin-binding proteins were under the control of a single gene. The riboflavin content of hen's egg albumen is related to that present in the diet (Heiman, 1935; Norris and Bauernfiend, 1940) although this does not apply to all avian eggs: the diet of ducks, geese and Adelie penguins may contain high levels of riboflavin but they do not secrete it in the egg albumen (Rhodes et al., 1959; Feeney et al, 1968), possibly because they lack the ability to synthesise the apoprotein in the oviduct.

The apoprotein molecule contains a large number of aspartate and glutamate amino acid residues (Tranter, 1982) and is cross-linked with eight disulphide bridges (Farrell et al., 1969). The apoprotein contains 14% carbohydrate as mannose and glucosamine

(Farrell et al., 1969). At pH 4.2 the flavoprotein molecule dissociates, but it will recombine above this pH and, in most biological situations, the binding of riboflavin is not influenced by pH.

In vivo the ratio of apoprotein molecules to riboflavin molecules is approximately 2:1 (Baker, 1968), yet Rhodes et al. (1959) had to establish a ratio of 10:1 in order to inhibit the growth (in vitro) of the riboflavin-dependant organism Lactobacillus casei. Apoprotein in ovo may be a part of an integrated antimicrobial system (the subject of a major review by Tranter and Board, 1982a), such that the stress imposed by the other antimicrobial proteins may dramatically reduce an organisms ability to take up riboflavin thereby resulting in inhibition.

Avidin

The biotin-binding protein, avidin, accounts for a maximum of 0.05% of the total protein in avian eggs. Avidin is not unique to the avian egg. It has been shown to occur in the jelly of frog's eggs and the oviduct of laying hens (Fraps et al., 1943; Green, 1975) and frogs (Hertz and Sebrell, 1942). It was this ability to complex with the vitamin biotin and render it unavailable that led to avidins discovery (Boas, 1927).

Avidin is a basic glycoprotein (MW 67,000) consisting of 128 amino acid residues, mannose and glucosamine components (De Lange and Huang, 1971). As with ovotransferrin, the avidin complex (one mole of avidin to four moles of biotin) is more stable than the inhibitor alone, although even the unsaturated molecule has a marked stability to denaturation by heat, pH or breakdown by proteolytic organisms (Wooley and Longsworth, 1942;

Gyorgi and Rose, 1943). Tai and Lichstein (1964) and Wei and Wright (1964) found that biotin was released more rapidly in the absence of salts yet even so vitamin release was only 88% complete after 10 minutes at 100°C (15 minutes at 120°C in the presence of salts). Avidin differs therefore from the saturated biotin-binding proteins of the chicken plasma and yolk which exchange biotin freely at body temperature (Mandella et al., 1978). Avidin and the biotin binding protein of the yolk/serum also have notably different isoelectric points - pI 10 and 5 respectively - (Mandella et al., 1978).

Roepke and Bushnell (1936) were the first to suggest that chicken egg yolk proteins were derived directly from the serum. Several studies (Maw, 1954; Williams, 1962; Schjeide et al., 1963; Winter et al., 1967) support the hypothesis that complexing proteins transport essential vitamins from the serum to the developing yolk. The biological function of avidin is however still unknown. An avidin-like protein has been isolated from the culture filtrate of several species of Streptomyces (Chaiet et al., 1963) and has consequently been named streptavidin. Green (1975) suggested that considering the great versatility of the streptomyces in devising antibiotic systems, streptavidin might support the contention that avidin's function is antibacterial, although evidence for this is extremely circumstantial. Tranter and Board (1982a) contested that too much attention has been focused on the microorganisms isolated from addled eggs and not enough on the role of the chemical defence in protecting the embryo and food reserve during embryogenesis. There is a need to determine the origin of these potentially antibacterial proteins and to elucidate their function(s).

Effect of temperature, pH and ovotransferrin saturation on bacterial growth

There have been only two major studies on the effect of incubation temperature on microbial development within the albumen. Ayres and Taylor (1956) studied the changes in the microbial population of albumen incubated over a range (2, 10 and 20°C) of temperatures while Tranter and Board (1984) studied the synthesis and effectiveness of enterochelin at various alkaline pH's and temperatures.

Tranter and Board (1984) reported that all the Gram-positive bacteria tested (Bacillus, Streptococcus, Staphylococcus, Micrococcus, Brochothrix and Kurthia spp.) were killed by egg albumen during the course of the 24h (30 and 39.5°C) incubation period. The saturation of ovotransferrin did not appear to influence the outcome. Death could not be attributed entirely to lysozyme action as two of the organisms (B. cereus T and M. luteus) were known to be resistant to the enzyme. Of the Gram-negative bacteria tested (Escherichia, Salmonella, Enterobacter, Proteus, Pseudomonas, Alcaligenes, Serratia and Acinetobacter spp.) only S. dublin multiplied in non-Fe^{3+}-supplemented albumen but only at the lower incubation temperature (30°C). Saturating the ovotransferrin with Fe^{3+} resulted in growth of all species apart from Acinetobacter spp.

Tranter and Board (1984) presumed that the bacteria which grew in unsupplemented albumen at 30°C possessed a specific iron-scavenging mechanism. At the higher incubation temperature (39.5°C), however, the effectiveness of iron-chelating agents is reduced. Garibaldi (1972) reported that incubation near to the upper temperature limit resulted in a decrease in the synthesis of iron-chelating agents. Tranter and Board (1984) suggested that this

might account for their observations.

Immediately following oviposition the pH of the hen's egg albumen is about 7.6 - 7.9, which is near the optimum for growth of most saprophytes. The pH does not remain at this level however, the loss of carbon dioxide from the egg causing a gradual increase in pH. One week after oviposition the albumen's pH has risen to about 9.5 (Healey and Peter, 1925) where it remains, unless gross contamination of the albumen occurs (the decrease in the albumen's glucose content, causing the pH to move back towards neutrality - Board, 1964).

The results of early studies (Parascandoles, 1893; Turro, 1902; Maurer, 1911; Rettger and Sperry, 1912) on the bactericidal nature of hen egg albumen tend to be very conflicting. This is a direct result of the failure to appreciate that the albumen pH changed with time and that this change might be very rapid (up to one pH unit in 24h, Sharp and Whitaker, 1927). Sharp and Whitaker (1927) investigated the effect of pH on the growth of the common contaminants of rotten eggs in incubated (37°C) albumen. They found that significant multiplication occurred in the 6h following inoculation of albumen adjusted to pH 6 - 8 but that organisms were killed in albumen adjusted to pH 9 - 10. Similar results were reported by Tranter and Board (1982b) and Banwart and Ayres (1957) who studied the effect of pH on Salmonella growth in albumen.

Sharp and Whitaker (1927) made the important observation that "the limiting hydroxyl ion concentration for the growth of bacteria varies with the components of the medium". This was demonstrated by Tranter and Board (1984) who incubated strains of E. coli in albumen held at 39.5°C. They reported that ovotransferrin-saturation allowed the organisms to grow at both pH 8.8 and 7.5 whereas

"unsaturated" albumen was bactericidal to these organisms at the higher pH levels.

Although Sharp and Whitaker (1927) concluded that pH *per se* played only a minor role in preventing Gram-negative organisms multiplying in the albumen, the effect of increasing pH on the growth of contaminants cannot be assessed independently of the eggs other antimicrobial components. The stress imposed by the albumen's high pH may limit an organism siderophore production and result in its death/stasis through iron deprivation - an effect that could not be brought about by ovotransferrin or albumen pH alone. This principle applies to all aspects of the albumen's antibacterial system. There is a need to understand, or at least appreciate, the integrated working of the albumen's chemical defence system, a view expressed recently by Tranter and Board (1982a).

Board and Fuller (1974) posed the question, "does the albumen change during development of the embryo so that its bacteriostatic properties are enhanced or diminished"? Lutsky and Bell (1953) indicated that the albumen of eggs that had been incubated for one or two days was bactericidally superior to unincubated eggs.

The passage of water from the albumen to the sub-embryonic fluid reduces the albumen's water activity but there are no indications (Board and Halls, 1973c) that the A_w is reduced sufficiently to impede the growth of Gram-negative bacteria.

It can be seen from this review of the literature that the avian egg makes many diverse contributions to the successful development of the embryo (exchange of respiratory gases, antimicrobial defence, etc) and that it has been the subject of many studies over the past 100 years. Unfortunately many of these studies

have been hindered by the lack of accurate and detailed knowledge concerning the egg's chemical and physical structure. It is beginning to be appreciated for example, that the cuticle plays an important role in protecting the egg from bacterial penetration, yet the cells responsible for cuticle production have not been identified, there have been no reports on any physical changes that might occur as the cuticle "dries out" post-oviposition and the reasons for cuticle-less eggs or the effect of "clutch-position" on the quality of the cuticle are subject to conjecture. Similarly water loss during incubation has been the subject of numerous studies, yet although a fundamental measure of water loss (G_{H_2O}) has now been widely adopted, the physical structures through which the vapour must pass have received relatively scant attention (the notable exceptions being the studies of Tyler, Tullett and Board). The determinants of pore number, distribution and size are still unknown, as is the mechanism by which the pore canal remains free from obstruction during shell formation.

The literature concerning the shell integument's physical structure can be seen to be somewhat fragmentary and was mainly carried out prior to the appearance of the latest generation of electron microscopes. The shell integument was therefore reexamined in this study using some of the most modern and powerful EM techniques currently available. The study falls into two sections; the consolidation of observations from earlier workers and the in depth examination of new observations concerning specifically the development of the cuticle, shell membranes and limiting membrane. Using this work as the basis for further study an attempt was made to shed new light on some of the questions raised above and on other related features of the avian egg and the apparently contradictory

demands made upon it (eg water conservation/gaseous diffusion).

MATERIALS AND METHODS

THE RESISTANCE NETWORK - FUNCTION

MATERIALS AND METHODS

Eggs

Infertile eggs (Ross Ranger) were obtained from hens housed in a commercial battery unit while fertile eggs were supplied by a commercial hatchery. The eggs were used immediately or incubated at 37.5°C/r.h. 60% in a small incubator (Brinsea Products Ltd., West Brinsea, Congresbury, Avon). The incubator automatically turned the eggs every hour.

Electron microscopy

Samples for SEM and TEM were prepared as described on pp 21.

INTEGUMENT POROSITY (K_{O_2}, G_{H_2O})

Water vapour conductance

Water loss from the egg was expressed as G_{H_2O}. G_{H_2O} is directly related to the number and size of pores in the eggshell and is a constant once shell formation is complete. Values were calculated according to the equation of Ar *et al.* (1974):

$$G_{H_2O} = \frac{\dot{M}_{H_2O}}{P_{H_2O}}$$

where G_{H_2O} = Water vapour conductance of the eggshell expressed as mg of water lost per mmHg difference in water vapour pressure across the shell per day.

$\dot{M}_{H_2O}$ = Water loss from the egg in mg per day.

P_{H_2O} = Difference in water vapour pressure across the

shell in mmHg.

G_{H_2O} was determined by packing pre-weighed eggs in desiccant, Drierite 10/20 mesh (Koch Light Laboratories Ltd., Colnbrook, Bucks, England), contained in a vented desiccator maintained at 37°C. The eggs were re-packed in fresh desiccant daily and re-weighed on day three. As the water vapour pressure around the eggs was 0 mmHg and the egg contents were assumed to be at the saturated vapour pressure (for the relevant temperature), P_{H_2O} may be determined and subsequently G_{H_2O}.

Measurement of oxygen permeability (K_{O_2})

The oxygen flux across the egg integument (the porous calcitic shell lined on its outer surface with cuticle and on its inner surface with fibrous membranes) of both fertile and infertile hen eggs was measured using apparatus (Fig. 7) described by Kutchai and Steen (1971).

Eggs were taken at various stages of incubation, cut around the shoulder using a diamond-tipped cutting wheel and the narrow pole carefully wiped clean of adhering albumen with a tissue. The shell was immediately sealed onto the apparatus with "Xantoprene" polymer (Bayer Dental Ltd., Leverkusen, Germany). The enclosed space was flushed with humidified nitrogen until the oxygen concentration - measured by an O_2 probe/meter (Walden Precision Apparatus Ltd., Saffron Walden, Essex) - was zero. The gas inlet and outlet were closed and the time taken to reach half the maximum O_2 concentration recorded on a chart recorder.

The Xantoprene-covered area of shell was removed and the surface area of the eggs determined by passing small (<0.25 cm^2)

pieces of shell through a surface area recorder (Lamda Instruments Corporation, Lincoln, Nebraska, USA).

The results were expressed as described by Wangensteen et al. (1970/1971).

$$K_{O_2} = \frac{(0.249)V}{t_{\frac{1}{2}}AT} \text{ cm}^3.\text{STP.sec}^{-1}.\text{cm}^{-2}.\text{mmHg}^{-1}$$

where: K_{O_2} = Permeability of the shell

V = Enclosed volume (cm^3)

t = Time (secs)

A = Area of shell (cm^2)

T = Absolute temperature

IRON-BINDING - SHELL MEMBRANES

The persistence and degree of iron contamination on the shell membranes was examined in two ways: a hole was drilled through the shell into the air space and 0.1 ml of the iron solution inoculated onto the outer surface of the inner shell membrane or, warm eggs ($37^{\circ}C$) were immersed in a cold ($4^{\circ}C$) iron solution, causing the suspension to be drawn down the pore canals onto the shell membranes. Iron contamination was assessed by TEM and chemical means.

A hypodermic syringe was used to aseptically inoculate 0.1 ml of a 6 mg/ml solution of filter (0.45 µm) sterilised $Fe(NH)_2(SO_4)_4.6H_2O$ into the air space. The air space was then sealed with paraffin wax. Eggs were dipped in a colloidal iron suspension prepared according to the method described by Rinehart and Abul-Haj (1951) and stored at $4^{\circ}C$.

All the glassware was freed from iron by the following procedure:

a) soak in 0.1M HCl for 14h; b) rinse in glass-distilled water; c) wash in 8-hydroxyquinoline (5 g/l) in chloroform (to chelate any remaining iron, Waring and Werkman, 1942); d) remove chelate by washing in chloroform; e) remove chloroform by drying in a hot air oven; f) rinse twice in glass-distilled water and dry in a hot air oven.

The iron concentration was determined as follows. Residual albumen was removed from the shell membranes by repeated rinsing with deionised water, then the membranes were solubulised in ammonium persulphate (20 g/l). Albumen was treated with an equal volume of ascorbate (0.2 g/l) in 0.2M HCl and allowed to stand for ten minutes. The protein was precipitated (using trichloroacetic acid - 110 g/l) and removed by centrifugation (5,000 g). Ammonium acetate (0.4 ml, 100 g/l) was added to 0.5 ml of either the solubulised membrane or the albumen supernatant. To this 0.1 ml of an iron reagent (Sigma Chemical Co., Poole, Dorset) consisting of 3 mg/ml ferrozine and neocuproine was added. The absorbance of the magenta-coloured complex was measured at 562 nm (water blanked) and the final iron concentration calculated from a calibration curve (constructed from testing known quantities of iron).

CUTICLE

Cuticle staining, removal and weight

As described on pp 22.

Cuticle crack length

As described on pp 23.

Water uptake - temperature differential

A modified technique based on the principles of Haines and Moran (1940) was used. Pre-weighed eggs (37°C) were immersed in a chilled (4°C) 1% (w/v) solution of Edicol Supra Pea Green H (ICI, Hexagon House, Blackley, Manchester, England). After 15 minutes the eggs were removed, dried by wiping with a paper tissue and weighed.

The cuticle was removed (by soaking in 10% NaOH for 15 minutes) from some eggs in order to measure the performance of "cuticle-less" eggs. Other than removing the cuticle there was no evidence of G_{H_2O} values being affected and an EM examination of the shell and pores indicated that no damage was caused to the shell membranes or pore canals.

The change in the "egg core temperature" once the egg was immersed in the chilled solution was also monitored. A small (<1 mm^2) hole was cut in the shell, leaving the underlying membranes intact. A "suba-seal" No.25 (Fisons Scientific Equipment, Loughborough, Leicestershire) was "araldited" over the hole forming a gas-tight seal. The egg was then warmed to 37°C and weighed as described above. Immediately prior to immersion a temperature probe (Edale Thermometer Model C - Edale Instruments, Toft, Cambridge) was inserted through the suba-seal/shell/shell membranes into the yolk. The change in temperature was monitored every 30 seconds for 15 minutes.

Water uptake - using a degassed solution

Excess gas was removed from the dip solution by maintaining a headspace vacuum of 750 mmHg for 48h prior to egg immersion.

Water uptake - application of pressure/vacuum

The eggs (at ambiant temperature) were immersed in water within an airtight container and a vacuum (750 mmHg) was applied to the headspace. After 30 minutes the vacuum was released, the eggs left for a further 15 minutes and then removed, dried and weighed.

Alternatively a pressure (69 kPa) was applied to the headspace for 15 minutes after which the eggs were removed, dried and weighed.

Water uptake - surfactant

The surfactant Triton X-100 (Sigma Chimical Co., Poole, Dorset) was added (0.05% v/v) to either the water or dye solution (used in the temperature differential dip) in order to lower the surface tension.

PORE AND CONE DISTRIBUTION

Pore counts

Tyler's (1953) method of etching the pores with concentrated nitric acid was adapted so that a field of 1 cm^2 of shell was observed (cf Tyler's field of 1 mm^2). This involved etching a shell fragment (approx. 1.5 cm^2) and mounting it on a projector slide over a 1 cm^2 aperture. When the slide was projected, the light spots corresponding to pores were superimposed onto paper and the pore number and distribution calculated.

Pore number per cm^2 was multiplied by the egg surface area to give pore number per egg. The surface area of the egg was calculated from egg weight (see Tullett, 1976);

$$\text{Surface area} = 4.917W^{0.661}$$

where W is the fresh egg weight (g).

There was a close correlation ($p < 0.001$) between the surface area calculated using the surface area recorder (described on pp 121) and those calculated using the above equation. Fresh egg weight was used to calculate surface area therefore, as it was as accurate as the previous method and less time consuming.

Pore distribution

The method of Tyler and Fowler (1978) was used, involving the measurement of nearest neighbours (r_A) in a known area of shell (A). From the mean ($\bar{r}_A$), a value Q' was determined:

$$Q' = \frac{A}{4(\bar{r}_A)^2}$$

When Q' is plotted against N (number of cones or pores), a random distribution will give a line bisecting the origins of the axis with a slope of 1.00. Points above this line are taken as evidence of aggregation whilst those below of uniformity. A line with a slope of 0.28 representing perfectly uniform distribution of close packed regular hexagons.

Cone counts and distribution

Pieces of shell removed from the shoulder of the egg were boiled in 2.5% (w/v) sodium hydroxide for five minutes to remove the membranes, rinsed thoroughly and left to dry. They were freeze dried for SEM viewing. Micrographs taken of the cone layer (area of 0.0013 cm^2) were analysed by means of the nearest neighbour equation described above.

Pore models

Pieces of dry shell from which the cuticle and membranes had been removed (as above) were dipped in ethyl acetate, and then immersed in catalysed resin (Plasticraft, Turner Research Ltd., Leeds). The ethyl acetate served to aid resin uptake by the pores. The shells were infiltrated with resin by repeatedly drawing and releasing a vacuum over the resin. Following polymerisation (60°C/24h) the resin block was cut back with "wet and dry" glass-paper to expose the outer surface of the shell. The shell was decalcified with concentrated hydrochloric acid, washed first in 10% (w/v) sodium hydroxide and finally in tap water. The resin blocks were then sputter coated as described above and viewed by SEM.

Shell thickness

An anvil-jawed micrometer (hemispherical jaws) was used to measure the thickness of shells from which the membranes had been removed. The mean of three readings, obtained from separate shoulder pieces was used.

BACTERIAL CHALLENGE OF THE "WET" AND "DRY" CUTICULAR STATES

Six hens (Light Sussex x Rhode Island Red) - in lay for 11 months - were individually housed in traditional roll-away battery cages. The birds were observed and immediately an egg was oviposited it was placed onto fresh chicken faeces for 15 minutes. The egg was then rotated and the opposing, now dry, side was then placed onto fresh faeces for 15 minutes, thus each egg possessed its own unique control.

The contents of the egg were then aseptically removed through

a sterilised 1.5 cm dia. hole cut in the sharp pole, any albumen adhering to the membranes was removed by washing with sterile ¼ strength Ringers solution. The egg was filled (Board and Board, 1967) with molten Nutrient agar containing 0.1% (g/l) of a tetrazolium salt - 2:3:5-triphenyl-tetrazolium chloride (BDH Chemicals Ltd., Poole, England) - indicator dye and when the agar had set the hole in the shell was sealed with sterile molten paraffin wax. Following incubation (37°C/24h) the egg was cut around a longitude to release the Nutrient agar block. Bacterial penetration was indicated by purple dye spots on the membrane and the agar block.

BACTERIAL CHALLENGE IN THE INCUBATING INFERTILE EGG

Inoculum

Fresh (<24 h) chicken faeces (200 g) were mixed with sterile ¼ strength Ringers solution (100 ml), 10 ml of this suspension was removed aseptically and diluted serially in sterile ¼ strength Ringers solution. Bristol black (Phil Black, Avonmouth, Bristol), a fine carbon powder, was added to the suspension to provide visual evidence of inoculation of the membranes.

The shell above the air space was sanitised by wiping with 70% IMS (v/v) and a diamond-tipped cutting wheel used to cut a small hole in the shell. The membranes were pierced with a sterile scapel and 0.02 ml of inoculum was placed on the inner membrane of the air cell. The hole was sealed with sterile molten paraffin wax.

Salmonella sp were added to some of the faecal inocula. The Salmonella were grown up in Trytone soy broth held at 37°C/18 h in a shaking water bath. Ten millilitres of broth were centrifuged (1000g) and the resultant pellet of cells washed in two changes of

sterile ¼ strength Ringers solution. The final cell suspension was serially diluted (¼ strength Ringers solution) to the required level and 1 ml added to the faecal suspension.

The Salmonella sp were stored on nutrient agar slopes at 4°C.

Incubation

Inoculated eggs were placed in a small incubator (Brinsea Products Ltd., West Brinsea, Congresbury, Avon) maintained at 37.5°C/r.h. 60% and turned by hand four times a day or they were "stored" in an incubator (15°C) for 7 days before being placed in the 37.5°C/r.h. 60% regime

Sampling

On each sampling day over the experimental period (up to 19 days) five eggs were removed from the incubator. The shells were sanitised by wiping with 70% (v/v) IMS and then a cutting wheel was used to separate the shell into two halves around the shoulder. A sterile scapel was used to cut the membranes and the egg contents were tipped into a sterile Petri dish from which 5 ml of albumen was withdrawn, this volume from each of the eggs being mixed together. A 1 ml sample was taken from the bulked sample and serially diluted in sterile ¼ strength Ringers solution.

The inner shell membrane was washed free of adhering albumen with sterile ¼ strength Ringers solution. The inner membrane lining the air cell of eight of the eggs was severed with a scapel and placed in a sterile glass mortar. The membranes from the eggs were ground in 10 ml sterile ¼ strength Ringers solution and a small amount of sterile sand. A sample (1 ml) of the finely suspended membranes was serially diluted in 9 ml of sterile ¼ strength Ringers

solution. Twelve drops (0.02 ml) of each dilution were placed individually on the dried surface of the appropriate agar and incubated for 24 h at 37°C.

The membranes from the remaining two eggs were used as specimens for transmission EM (method of preparation as described on pp 21.

Media

Six media were used to analyse the microflora of the membranes:

1. Nutrient agar - General count (Board, 1964, Board and Ayres, 1965, Seviour and Board, 1972, Anon., 1982c, Tranter and Board, 1984).
2. Nutrient agar + 6% NaCl - Micrococci (Seviour and Board. 1972).
3. Kanamycin Aesculin Azide agar - Group D Streptococci (Mossel et al., 1976, Anon., 1982c).
4. Pseudomonas agar base/C-F-C supplement - Pseudomonas (Mead and Adams, 1977).
5. Violet Red Bile Glucose agar - Coliforms (Board et al., 1964, Seviour and Board, 1972, Anon., 1982c).
6. Desoxycholate Citrate agar - Salmonellae (Ostlund, 1971, Anon., 1982c).

The micro-organisms in the albumen were isolated on:
Brain Heart Infusion agar - General count (Barnes and Corry, 1969, Payne et al., 1979).

All the media was manufactured by Oxoid Ltd., Basingstoke, Hants.

Typical colony types were characterized by Gram-staining and the API 20 E system (API System SA, La Balme Les Grottes, 38390 Montalieu Vercieu, France).

Salmonella CHALLENGE AND FATE IN INCUBATED ALBUMEN

Salmonella inoculum

The inoculum was prepared as described on pp 128.

Albumen

The eggs were swabbed with 70% (v/v) IMS, the shells cracked and the contents poured into a sterile Petri dish. A wide bore pippette was used to harvest albumen, the albumen of several eggs being mixed in a screw-capped bottle by gentle shaking.

Sampling

A pre-determined inocula was added (1 ml) to 50 ml of albumen in 250 ml Erlenmeyer flasks which were incubated in a shaking water bath at the temperatures noted in the text. At appropriate time intervals 1 ml of albumen was withdrawn and serially diluted in 9 ml amounts of sterile $\frac{1}{4}$ strength Ringers solution. Sixteen drops (0.02 ml) of each dilution were placed individually on the dried surface of Plate Count agar (lab M Ltd., Salford, Lancs.) and the colonies counted after incubation for 24 h at 37°C.

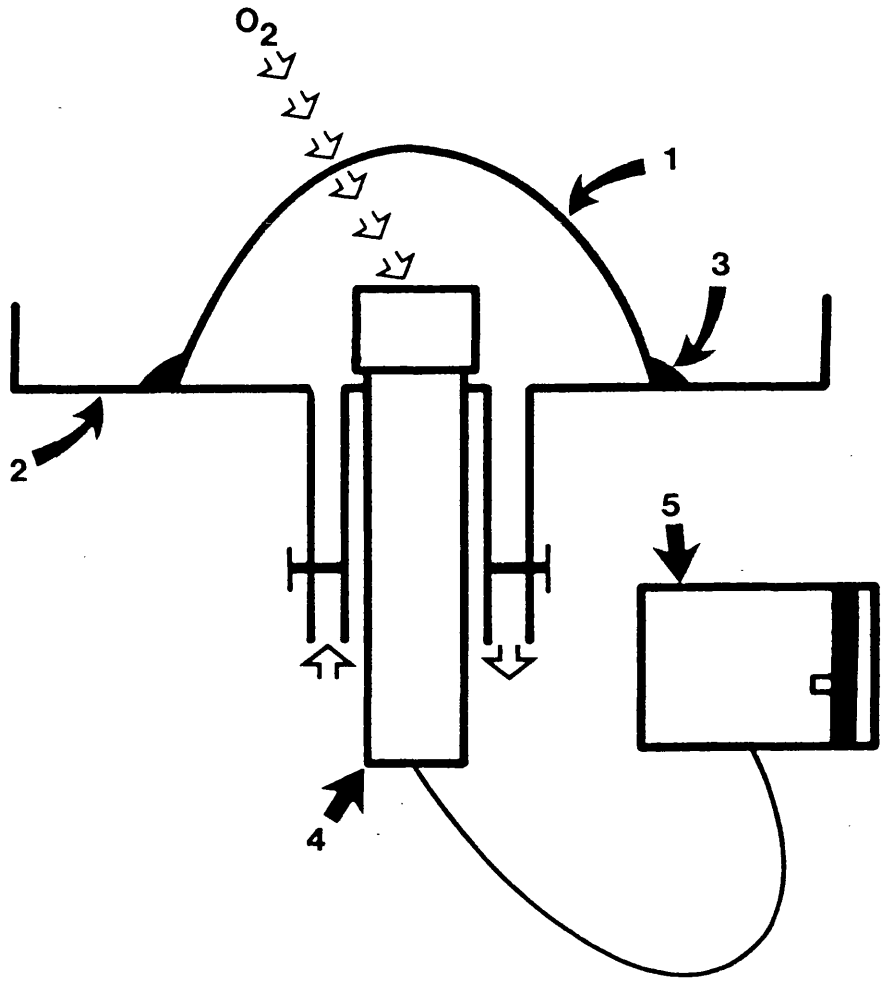


Fig. 7 Schematic diagram of the apparatus (adapted from Kutchai and Steen, 1971) used to measure the oxygen permeability of an egg shell. The sharp pole of the egg (1) was sealed onto the modified glass petri dish (2) with "Xantoprene" polymer (3). The oxygen electrode (4) was connected, via a meter, to a chart recorder (5).

RESULTS

RESULTS

The reasons for using water/dye uptake as an index of bacterial penetration studies were firstly that Board and Halls (1973a) showed that the pores penetrated by liquids were equally susceptible to bacterial penetration and secondly it is considerably easier to work with a coloured fluid as opposed to the sterile techniques required for bacterial studies. Moreover the use of dyes provides immediate information about the location and degree of penetration that occurs.

The extent to which the integument is penetrated depends on the resistance offered (in series) by each of its components - the concept of the shell integument acting as a resistance network (Fig. 1). Therefore, although components may be studied in isolation, ultimately they must be considered as part of the complete integument. The cuticle is the first resistance in the domestic hen's egg to liquid/bacterial penetration.

CUTICLE

Effect of cuticle maturity

A small number of studies have suggested (as discussed in the Literature Review) that the moist, recently-oviposited egg is more susceptible to penetration than its "dry" counterpart.

The electron microscope study (Plate 2) indicated that the cuticle underwent a rapid maturation process as it dried (pp 29). It was postulated that the relatively open structure of the immature or "wet" cuticle would offer less resistance to bacterial penetration

compared with that of the "dry" cuticle. Initial tests using a sponge soaked in a 1% solution of the dye Edicol Supra Pea Green H supported this supposition. The observations were repeated therefore using fresh faeces. In practice, immediately following oviposition, eggs were placed against faecal material for 15 minutes. They were then reversed so that the dry cuticle rested against the faeces. Of the 12 brown eggs tested, all the immature cuticles as well as pores in the underlying shell were penetrated by bacteria, as shown by bacterial growth in the membranes. Only two mature cuticles were penetrated. The extent of penetration through the mature cuticle was always minimal (two or less spots of the insoluble dye, formazan) compared (Fig. 8) with a minimum of three spots under the immature cuticle (Plate 19). It is notable that the eggs infected via the dry cuticle showed an above average incidence of penetration in the wet state also. This suggests that cuticle quality has been determined before the cuticle dries post-oviposition, but this aspect was not examined. Even very careful handling of a freshly oviposited egg resulted in the removal and disruption of the immature cuticle. Indeed this probably accounts for the scratches seen on so many eggs laid in cages.

Two factors restricted severely the number of eggs included in this study, bearing in mind that the eggs had to be collected and placed against the faeces within seconds of oviposition as the cuticle dried very rapidly at the temperatures in the battery unit. Six birds, individually caged, were found to be the maximum that one person could competently observe. This posed the first restraint. The second resulted from birds sitting on their eggs post-laying, thereby allowing the cuticle to dry before the eggs could be examined.

Mature cuticle

A differential temperature (eggs at 37°C, fluid at 4°C) dipping technique was used extensively during this stage of the study as a means of measuring the ability of the shell integument, in particular the cuticle, to restrict fluid uptake by the egg. Preliminary studies were undertaken with a wide range of egg weights and cuticle types to ensure that this technique would not favour a particular egg weight/cuticle type. They showed that the bulk of the water was taken up within the first five minutes of immersion (Fig. 9a, b, c, d) and that the rate of the change in the core temperature of the egg - caused by the warm egg being placed in cold fluid - was identical ($p < 0.001$) for all eggs in the fresh weight category under test. The technique employed did not apparently introduce any artefacts.

It is evident from Fig. 10 that the cuticle on the shell of fresh (ca. 24h) brown eggs offered greater resistance than that of fresh white eggs to water penetration when warm eggs were placed in cold dye solutions. Storing brown eggs for three days in "drierite" (37°C/r.h.0%) caused a marked diminution in the resistance of the egg to water uptake although SEM studies did not show any changes in the cuticle. The size of the air cell, which was enlarged by water lost to the drierite, is important therefore when considering fluid uptake. The force exerted when the larger air cell of the "stored" eggs contracted was sufficient to take up to ten times the amount of fluid taken up by the "fresh" eggs. It was for this reason that day-old eggs only were used in this study.

The role of the cuticle on brown eggs in impeding the passage of fluid was examined in more detail by removing the cuticle (using EDTA) from some eggs. The eggs were then warmed to 37°C and

plunged into ice cold dye solution. There was an appreciable increase (Fig. 11) in water uptake by the EDTA treated eggs compared with the control group. As the G_{H_2O} (water vapour lost by conductance) values for the EDTA treated group did not differ significantly ($p > 0.05$) from the controls, it was concluded that the chelating agent had not modified appreciably the pore canals. The increased uptake could therefore be attributed solely to the removal of the cuticle. This was reflected also in naturally occurring partially cuticle-less eggs which were identified by their poor uptake of stain. Eggs which fell into this category tended to have either a cuticle-less pole or a thin/malformed cuticle over the entire shell. Differential temperature dipping such eggs in dye solution resulted in gross staining of the membranes underlying the affected area of shell. Thus this phase of the study showed that lack of cuticle, or more correctly, the associated pore plug, was the most important cause of water penetration of the underlying pores.

Having determined the importance of the cuticle (and in particular the brown cuticle) in restricting fluid penetration of the shell, attempts were made to correlate particular features of the cuticle with water uptake. This would allow a cuticle to be measured in terms of quality as opposed to simply being quantified.

As the cuticle on brown eggs never had a uniform thickness over the shell surface and their fissured surface was partially covered by solid "plaques" (Plate 4) absorbance measurements of cuticles stripped by EDTA from the shell were used in an attempt to circumvent these problems. There was no correlation, however, between the absorbance (383 nm) of the stained cuticle suspended in water and water uptake (number of eggs, 50). The dry weight of

cuticle (average weight 1.33×10^{-3} g/cm^2, see Fig. 3) taken from brown eggs (number of eggs, 101), was also found not to be associated with the amount of water taken up by the eggs. Beside the lack of plaques and extensive fissuring in the cuticle of white eggs (Plate 3), a limited survey (60 eggs) showed that the cuticle on such eggs averaged 35% less in terms of dry weight/cm^2 (average weight 4.60×10^{-4} g/cm^2). Both these features almost certainly account in part for the decreased resistance to fluid uptake that was found in white eggs (Fig. 10).

The amount of fissuring (cracking) of the cuticle was a notable feature of the cuticle of both white and brown eggs when viewed with the SEM (Plate 3). It was deduced that anything passing through the integument must pass through these fissures. To determine if the degree of cracking affected water uptake, and thus could be used to index the cuticle's potential for water resistance, the following procedure was adopted. The water uptake values were determined for 50 brown eggs, from which five eggs were selected as representatives of those having high, intermediate or low water uptake. The total length of the fissures per egg was measured (see Materials and Methods pp 23). In practice the length varied from 12 cm to 32 cm/cm^2 (average 23 cm/cm^2, see Fig. 4). There was a significant ($p<0.05$) linear correlation ($r = 0.93$) between crack length and water uptake (Fig. 12). This was found also for a second group of five eggs ($r = 0.85$, $p < 0.05$) taken from the batch of 50. These observations indicated that changes in one variable - crack length - accounted for 86% ($0.93^2 \times 100$) and 70% ($0.84^2 \times 100$) respectively of the changes in the other variable - water uptake in these specially selected eggs. (It

must be emphasised that very few eggs were observed, yet in both groups the regression coefficient was linear). No correlation was evident, however, when another 24 of the 50 eggs were examined for water uptake and crack length in the cuticle. White eggs were not studied as the fissuring was too extensive and could not be quantified by the technique used in this study.

It can be inferred, therefore, that the amount of liquid penetrating the shell integument is a function of many variables. The presence or absence of the cuticle being the most important one (Fig. 11). Another attribute is the degree of fissuring of the cuticle. When a small number of eggs from the representative group of 50 was examined the other as yet unidentified variables did not cause a significant influence on the relationship between crack length and water uptake. Increasing the number of eggs allowed the expression of the other variables and consequently there was no correlation between crack length and water uptake in the second, larger, group of eggs.

When water uptake was monitored with the dye Edicol Supra Pea Green H it was evident on breaking open the eggs (brown) that some which took up a relatively large amount of water (>100 mg, see Fig. 11) were only penetrated at one or two points. Microscopic observations of the external/internal shell surface commonly revealed shell deformation at the point of penetration. Several types of deformation were observed (see Table 3): calcareous lumps (EDAX analysis), hairline cracks, or large pore canals (Plate 20) - "Patent pores" (see Board, 1980) - a result probably of a localised malformation in the cone layer. Calcareous lumps were associated with gross penetration when they were embedded in the outer part of the calcitic shell and distorted a pore, or if they distorted

the cuticle/pore plug over a pore orifice. Many (approx 50%) calcareous lumps were associated solely with the cuticle; such lumps could easily be removed from the shell surface and they were not associated with abnormal penetration.

In summary, the following points can be made on this phase of the study:

1) The immature cuticle of the hen eggs having brown shells is more susceptible to bacterial and fluid penetration than the mature cuticle.
2) The cuticle on white eggs is less effective than the cuticle on brown eggs. The extensive fissuring on the cuticle of the former and the tendency for white eggs to have a lower ratio of cuticle (dry weight) to shell surface area (compared with brown eggs) almost certainly account for the relative inefficiency of the white cuticle as a resistance to water.
3) The cuticle is the main impediment to fluid penetration into the shell integument.
4) The size of the air cell is important in determining the amount of water taken up when a warm egg is immersed in a cool fluid. Thus age of the egg and storage conditions both affect the size of the air cell and must be given due consideration in practices that seek to introduce antimicrobial agents into hatching eggs.
5) The effectiveness of the cuticle in endowing the egg with water resistance is determined by several attributes (eg degree of fissuring, weight of cuticle/cm^2, percentage area of shell covered by cuticle). Thus while it is relatively easy to quantify the cuticle (in terms of weight per unit area) staining, although a subjective assessment, appears to be the only means available to poultry breeders and hatchery managers for the routine

assessment of cuticle quality.

6) The cuticle's efficiency may be negated by structural flaws such as cracks in the shell, large pores (the cuticle's mechanical properties not being sufficient to bridge the pore mouth?) and calcareous lumps.

PORE CANAL

The pore canal is the second resistance in the integument of the hens egg. The pore canals determine the shell's porosity - a nebulous term that has had many definitions. Recently G_{H_2O} (water vapour conductance) has been used as a measure of shell porosity and used to calculate the functional pore area (FPA). Using G_{H_2O} as a fundamental feature of the egg integument, the following questions were asked:

1) What is the relationship, if any, between G_{H_2O} and water uptake?
2) What is the relationship, if any, between G_{H_2O}/water uptake and pore number?
3) Is the pore distribution normal in eggs with extreme G_{H_2O}/water uptake values?

Water uptake/G_{H_2O} relationship

It has often been inferred or assumed that the shell's porosity offers an important constraint to fluid penetration through the shell integument. The results of the present study (number of eggs studied, 215) demonstrated that there was no correlation between G_{H_2O} (shell porosity) and water uptake (Fig. 11) when warm (37°C) eggs were immersed for 15 minutes in cold (4°C) water. The removal of the cuticle did not affect this situation.

Water uptake/G_{H_2O} and pore number

In the past pore numbers have often been used as a measure of shell porosity (Rizzo, 1899, Weston and Halnan, 1927, Almquist and Holst, 1931). It is evident from this study (Fig. 13), however, that there was no correlation between the number of pores per shell ($8 - 20 \times 10^3$) and water uptake ($r = 0.03$; $p > 0.05$; number of eggs 70) or the number of pores and G_{H_2O} ($r = 0.15$; $p > 0.05$; number of eggs 70).

The lack of correlation between pore number and G_{H_2O} is evidence presumably of the variability of pore configuration. If all the pores were of the same diameter, then there should be a highly significant correlation. The variety of pore configurations is evident in Plate 21. Pore number, therefore, is not a very useful parameter on its own and it must be linked to the total pore area. A functional pore area (FPA) may be calculated in one of two ways, using either fresh weight - $A_p = 9.2 \times 10^{-5}.W^{1.236}$ - or G_{H_2O} values - $A_p = 4.27 \times 10^{-4}.G_{H_2O}.L$ - (Ar et al., 1974). As would be expected, the relationship between FPA (calculated from G_{H_2O}) and G_{H_2O} values was highly significant ($p < 0.001$, 100 eggs). FPA (calculated from fresh weight) and G_{H_2O} values were not significantly related ($p > 0.05$, 100 eggs). Neither method of calculating FPA provided a significant relationship with water uptake (this applied to eggs with a normal cuticle as well as cuticle-less ones). The lack of a correlation between FPA (calculated from fresh weight) and G_{H_2O} suggests that while fresh weight may be used for interspecific studies (eg Ar et al., 1974), where marked differences will be found a feature, they have limited utility in intraspecific studies. FPA measurements should be derived from G_{H_2O} measurements in the latter instance. These results highlight a fundamental feature of

eggshells, namely that different impediments are imposed by the shell integument to water lost as a vapour or taken into the egg as a liquid. In other words, evolutionary pressures have selected for water conservation in one manner (pore configuration and pore number/cm^2) and water resistance in another (cuticle cover over the pore canal).

Pore distribution and G_{H_2O}/water uptake values

These features were evident also in studies of pore distribution. The distribution of the pores and cones was examined to see if the distribution was abnormal in shells associated with extreme G_{H_2O}/ water uptake values. The shells of eggs taken from clutch sequences were also examined to see if the distribution varied within or between the clutch.

The pore number ranged from 79 to 288 per cm^2 in a sample of 200 hens' eggs. Plotting pore number against pore distribution gave a correlation coefficient of +0.96 with a slope 0.85. It was concluded, therefore (as had Tyler and Fowler, 1978), that pore numbers are "highly predictive" for pore distribution. The distribution of pores tends (R = 1.1521) towards uniformity (see pp 126 for statistical details) although thay are by no means near to perfect uniformity (R = 1.00 - random distribution; R = 2.1491 - uniformity by regular hexagonal packing), again an observation in accord with Tyler and Fowler (1978).

Pore number and consequently distribution was found not only to vary in a random manner between birds but within the same clutch of eggs (Fig. 14). This variation within a clutch is unlikely to occur if the pore location is determined by the distribution, in utero, of the cells responsible for the secretion of the

carbohydrate cores (sites of cone initiation), as suggested by Wyburn et al. (1973). This random variation within a clutch is interesting as G_{H_2O} values were shown (Fig. 14) to increase and then decrease throughout the clutch.

Cone numbers varied from 184 to 757 per cm^2 (61 hen eggshells). The plot of cone number against cone distribution gave correlation coefficients of +0.95 (highly predictive), with a slope of 0.55 - cones showing greater uniformity (R = 1.5342) than the pores (R = 1.1521).

There was no significant correlation ($p > 0.05$) between cone and pore numbers or between shell thickness and cone number. Tullett (1976) found the former group to be significantly correlated, although this may have been because this author employed a different method to measure pore number. There was no evidence that the pores or, more particularly, the cones in shells with extreme water uptake/ G_{H_2O} values had significantly different distributions. Thus the two features noted above - water evaporation and water resistance - are not closely linked. If they were, then one would expect high porosity to be associated with marked water uptake. This is evidently not the case (see Fig. 11).

Having identified the cardinal role of the cuticle in water resistance, it is possible to consider factors that will amend its efficiency. Two such factors were examined, (1) reduction of tensile strength of water and (2) degassing of water.

Effect of treating dip solution

The use of a surfactant (Triton X-100) to lower the surface tension (Fig. 15) of the water was found to increase the mean weight by 344%. With such treatment, although some discrete spots

were seen, in most instances large patches of dye were present on the inner surface of the inner membrane. This is an expected consequence of the use of water with a low surface tension (Board and Halls, 1973a). By reducing the surface tension the "drag" exerted by the cuticle and probably by the pore canals also was reduced.

Degassing the water used in the differential temperature dip led to a significant ($p < 0.001$) increase in water uptake of the dipped eggs compared with the controls (Fig. 16). It was assumed that with the latter gas came out of solution thereby satisfying, in part, the pressure differential caused by the contraction of the contents of eggs subjected to a temperature differential dip.

In 1863 John Davey of Edinburgh (see Rahn et al., 1979) injected air into the air cell of a submerged egg. The evolution of gas from the shell demonstrated the location of the pore canals. Over 70 years later Bryant and Sharp (1934) modified this technique in an attempt to determine shell porosity and identify pores liable to bacterial penetration. They immersed eggs in water contained in a gas-tight container and applied a vacuum to the headspace. This caused the gas within the shell integument to be released as streams of bubbles. When the vacuum was released water was taken into the egg. This method was used in this study as well as a variation in which pressure was applied to the headspace to force liquid into the pore canal. Neither led to a significant ($p > 0.05$) increase in water uptake compared with the controls. As Bryant and Sharp (1934) were interested in counting pore numbers they did not record weight increase for the eggs under test (nor did they record the headspace vacuum). In both cases in the present work, the forces exerted (vacuum of 750 mmHg or a pressure of 69 kPa) were

insufficient to overcome the resistance imposed by the cuticle and pore canal. Pulling a vacuum in the headspace caused bubbles to emerge from the cuticle surface but when released it was insufficient to cause a significant influx of water.

Once contaminated fluid has penetrated the cuticle and overcome the drag imposed by the pore canal, bacteria may be located either within or on the outer surface of the shell membrane. Because of the mesh-like structure of the membranes it has been suggested (Haines and Moran, 1940) that they act as a physical barrier, thereby delaying bacterial contamination of the albumen. It has been suggested by Board (1966), however, that rather than the membranes preventing contamination of the albumen they "protect" bacteria from the albumen's various antimicrobial agents, in particular the major agent, ovotransferrin. In other words the membranes may be seen as offering a haven in which bacteria might subsist until favourable conditions for growth obtain. In this context, iron is of particular importance.

Iron transfer

Iron's ability to saturate ovotransferrin and thereby neutralise the latters bacteriostatic attribute would make it an important contaminant of wash water. The practical implications of such contamination were demonstrated by Garibaldi and Bayne (1962a, b) who found that there was a greater incidence of rots in eggs washed with iron-contaminated water than with those washed in water containing very little of this element. The role of the cuticle in impeding iron translocation was investigated. Warm eggs were immersed in cold solutions of colloidal iron. The iron breached the pores and stained the underlying shell membranes (Plate 22).

The cuticle was instrumental therefore in determining the amount of iron that passed onto the shell membranes because the element only entered vulnerable pores.

Board et al. (1968) found that iron-contaminated shell membranes encouraged the growth of bacteria that were previously quiescent and this resulted in a heavy contamination of the albumen. The studies of Garibaldi and Bayne (1962a, b) and Board et al. (1968) indicate that iron remains in the vicinity of the shell membranes, presumably in a form which can be readily utilised by the contaminants. Board et al. (1968) noted that iron staining of the membranes persisted during eight weeks storage of eggs at 10°C. Indeed the TEM study showed that iron added to the shell membranes impregnated the entire mantle of the fibre (Plate 22). It is evident from Fig. 17a that, during ten days incubation (37.5°C), the iron content of the inner membrane had diminished from 500 to 100 µg/ml of hydrolysate. The amount of iron diffusing into the albumen (Fig. 17b) - even at its peak concentration (150 µg/ml on day 17) - would have been insufficient to saturate the ovotransferrin in the underlying albumen. Thus it can be concluded that iron will follow the same course as bacteria in the process of egg infection, both being deposited on/in the shell membranes.

The fact that iron penetrates the entire mantle accounts not only for the staining noted by Board et al. (1968) but also this location ensures a source of this element for bacteria in the vicinity.

Shell membranes

The studies of the fine structure not only confirmed the

existence of the limiting membrane but identified also a potential barrier to bacterial infection of the albumen. As the limiting membrane is such a fine structure, evidence of its efficiency as a barrier was sought: transfer of respiratory gases being used for this purpose. The oxygen permeability of incubated fertile eggs was studied in conjunction with an SEM study of the structure of the integument.

Oxygen permeability

The integuments of 33 infertile chicken eggs averaged an oxygen permeability (K_{O_2}) of 1.0 x 10^{-7} $cm^{-3}O_2$ STP.sec^{-1}.cm^{-2}.$Torr^{-1}$ (Fig. 18) throughout incubation (37.5°C/r.h. 60% turned every hour). A similar value was obtained with the integuments of 24 fertile eggs until the fourth day of incubation when the resistance to oxygen diffusion decreased approximately ten fold (Fig. 18). At the tenth day of incubation they had attained a K_{O_2} of 1.0 x 10^{-6} $cm^{-3}O_2$ STP.sec^{-1}.cm^{-2}.$Torr^{-1}$, a level that in theory would have been sufficient to support continued embryonic development. During embryonic development the oxygen consumption increases rapidly between days ten and 14 of incubation to reach a plateau of 600 ml day^{-1}, compared with values of <50 ml day^{-1} at day four of incubation (Rahn et al., 1974, 1979).

Evaporative water loss was monitored throughout incubation to determine: (i) if the increase in permeability affected the diffusion of water vapour as well as that of the respiratory gases and (ii) to determine if the increased oxygen permeability was associated with an increased rate of water loss from the shell membranes. The latter has been suggested by several workers (see Kayar et al., 1981). This study has shown that the cuticle does

not significantly affect water vapour conductance and can be ignored in this context (see pp 141). Evaporative water loss from fertile and infertile eggs was found to be constant throughout incubation (Fig. 19), a result that agrees with the findings of Wangensteen et al. (1970/1971). Lomholt (1976) suggested that some of the water in the membranes, rather than being lost to the atmosphere, was drawn osmotically out of the inner membrane into the albumen. Kayar et al. (1981) noted however that filling eggs from early in incubation with albumen taken from eggs late in incubation did not lead to the 10 fold increase in oxygen permeability that would be predicted from Lomholt's (1976) hypothesis.

Wangensteen et al. (1970/1971) implied from a constant rate of water loss that the shell's permeability was also constant. The results of this study (Fig. 18) and others (Kutchai and Steen, 1971, Lomholt, 1976, Tullett and Board, 1976, Kayar et al., 1981) contradict this assumption - the permeability to respiratory gases increasing part-way through incubation. All the explanations offered for this increase depend however on the loss of water from the membranes. The electron microscope survey that accompanied this study identified another possible interpretation.

The fine structure of fertile and infertile shell membranes were identical (as described - pp 33), up to the fourth day of incubation (37.5°C/r.h. 60% turned every ½h). The infertile egg shell membranes remained unchanged up to day 14. That of a fertile egg showed signs of cracking on the fourth day of incubation. The degree of cracking increased as incubation proceeded (Plate 23b), the cracks getting longer and wider until by the 18th day of incubation (Plate 23c) the fibres underlying the limiting membrane were evident.

EM studies showed that this fracturing was related to the development of the chorio-allantoic membrane. This membrane makes contact with the limiting membrane on approximately day five of incubation and continues to establish its network of capillaries until day 12 it has covered the inner surface of the inner membrane (Rahn et al., 1979, Ackerman and Rahn, 1980). Kutchai and Steen (1971) and Kayar et al. (1981) postulated that chorio-allantoic formation might be related to the observed increase in permeability values in the incubated fertile egg. They contend, however, that it was the increased water loss from the inner shell membrane, promoted in an as yet undetermined way by the chorio-allantoic membrane, that resulted in the lowering of the integument's resistance. My observations indicate that the intimate contact of the limiting membrane with the chorio-allantoic membrane is a crucial and, up until now, overlooked factor in causing damage to the limiting membrane. Moreover the evidence does suggest a potential barrier to bacterial contamination of the underlying albumen, a topic discussed on pp 265.

BACTERIAL CHALLENGE IN THE INCUBATING HENS' EGG

The shell membranes are the location at which the egg's strategem against bacterial contamination appears to change from a physical defence to one in which chemical factors play an important part also. Although penetration by inert particles can be used to demonstrate the physical contribution of the membranes they do not, of course, provide evidence on the contribution of the chemical defence. Bacterial challenge experiments are therefore essential when studying the latter feature. Fresh chicken faeces were used

for the bacterial challenge experiments because evidence was sought on the selective forces, if any, acting on a mixed contamination of the shell membranes. A variety of organisms (Table 4) were isolated from fresh chicken faeces (obtained from birds housed in a commercial battery unit) and identified using microscopy, standard isolation techniques and the API 20E system (API SA, La Balme, les Grottes, 38390 Montalieu, Vercieu, France). No salmonellae were isolated from the faeces so these were added as required.

Faecal challenge *via* the air cell - fresh eggs

When a faecal suspension was inoculated onto the inner membrane of incubating (37.5°C/r.h. 60%) eggs - age at inoculation, one day - at levels of 10^2 cfu/membrane or less, they could not be isolated after two days (Fig. 20a). With initial inoculum levels of over 10^3 cfu/membrane the size of the total viable count of micro-organisms increased, the extent being related to the inoculum size (Fig. 20a). This trend was a feature also in the counts of coliforms (Fig. 20b), enterococci (Fig. 20c) and salmonellae (Fig. 20d). Inoculum size did not prevent the rapid diminution of the micrococci populations (Fig. 20e). Indeed these organisms were not isolated on the second day of incubation. The rate and extent of albumen contamination (Fig. 20f) also reflected the level of membrane contamination and hence size of inoculum. Thus no contamination of the albumen was detected after four days of incubation when the initial membrane inoculum was $<10^4$ cfu/membrane. Contamination was demonstrated after four days incubation when an inoculum size of 5×10^5 cfu/membrane was used (Fig. 20f). Increasing the size of the inoculum by a further log cycle caused an ultimate increase of just under two log units in the level of albumen contamination. These trends were evident in the

experiments summarised in Table 5. Thus in this phase of the study with incubation at 37.5°C following immediately on from inoculation of the air cell, the critical inoculum size appeared to be ca. 10^3 cfu/membrane. With only slightly smaller inocula the organisms failed to colonise, though whether through death or movement of micro-organisms into the underlying albumen was not determined. In order to gain additional information on what appears to be a critical inoculum level, incubation time was extended to 19 days.

Initially (Fig. 21a) the changes in the numbers of organisms within the membranes followed the pattern shown in Fig. 20a. The "growth curves" of the organisms (coliforms/Fig. 21b, enterococci/ Fig. 21c, pseudomonads/Fig. 21d, Salmonella hadar/Fig. 21e) that contributed to the inoculum tended to follow the same trend as the changes in the total counts, ie a rise in numbers over the first 2 - 3 days followed by a gradual decline. Only the micrococci deviated from this pattern with the number of cfu staying around the inoculum level. Paton and Ayres (1964) using a fluorescent brightener noted also multiplication of Salmonella within the shell membrane. Following the decline in the number of cfu after day three, there was a dramatic increase (between days 12 and 14) in the total number of viable organisms within the membrane (Fig. 21a) and a corresponding increase in the level of contamination of the albumen (Fig. 21g); it increased from an undetectable level to $>10^{10}$ cfu/ml. The results of other experiments, which showed these trends also, are presented in Table 6.

Increasing the inoculum size from ca. 10^3 to 10^6 cfu/membrane (Fig. 22, Table 7) did not affect dramatically the development of the contaminants in the membrane. The numbers of micrococci (Fig. 22f) rose initially and then fell to below detectable levels. The levels of faecal streptococci (Fig. 22c) rose initially and then fell,

increasing again as the albumen was colonised (Fig. 22g) - the rate and extent of albumen contamination was similar to that seen at the lower inoculum level (Fig. 21g). A low inoculum has therefore the potential, providing that the organisms remain viable during extended incubation, to cause the same level of albumen contamination that results from larger ones. The only notable difference found at the higher inoculum level was that the coliform and the total count in the membrane increased at a steady rate from the beginning to the end of incubation as opposed to the decline (between days 4 - 14) in cfu that was seen at the lower inoculum level. As in the experiments summarised in Fig. 20 and Table 5, the changes in numbers of Salmonella spp. (S. anatum, Fig 22e/Table 6; S. hadar, Fig. 21e/ Table 7; S. virchow, Table 6, 7) paralleled those of the total count curve. Thus there was no apparent antagonistic interaction between the Salmonella spp. and the endogenous faecal organisms.

Faecal challenge via the air cell - stored eggs.

Eggs are frequently stored for several days before setting. Indeed maximum hatch rates have been achieved by some (eg Jones, 1981) with eggs that were stored (16°C/r.h. 75%) for three days. During storage at 16°C/7 days (Fig. 23) the number of organisms in the membranes increased from 1.5×10^3 to 4.7×10^9 cfu/membrane, without detectable contamination of the albumen. Within two days of being transferred to the incubator at 37.5°C (r.h. 60%) the membrane count rose from 9.00×10^5 cfu/membrane to 3.00×10^8 cfu/membrane, with a concomitant increase in the numbers in the underlying albumen - from "no detectable count" at the time of transfer to 1.87×10^9 cfu/ml within 24 hours. It was notable that the high total count in the membrane was a transient feature,

the level falling by ca. one log cycle by the fourth day. The changes in the sizes of the particular groups of organisms (coliforms/Fig. 23b, enterococci/Fig. 23c, pseudomonas/Fig. 23d, Salmonella hadar/Fig. 23e, micrococci/Fig. 23g) tended to follow the trend indexed by the total count. One exception was noted with micrococci. The level of their counts (Fig. 23g) rose following transfer to the higher incubation temperature but thereafter they fell to below the initial inoculation level - the only count so to do. Another exception to the general trend was provided by the enterococci, whose number of cfu (Fig. 23c) gradually declined once the organisms were incubated at 37.5°C. In a subsequent study (Table 8), however, the "growth curve" conformed to the trend set by the total membrane count.

This study has perhaps identified a feature of commercial significance. At the temperature (16°C) used for storage of incubating eggs, microbial growth was restricted initially to the shell membranes. The same level of membrane contamination in eggs at 37.5°C caused contamination of the albumen. In either instance the membranes were not bactericidal to the organisms, other than micrococci, which occur in hens' faeces.

TEM survey of faecal challenge

This phase of the study was undertaken with the objective of establishing whether or not contaminants of the shell membranes could bring about structural change to the membranes and/or the limiting membrane. A faecal inoculum was mixed with carbon black. The latter was intended to identify the location of the inoculation site for TEM. The times of examination with the TEM (referred to in Plate 24 - 30) corresponded with those samples which were taken for

growth studies (Figs. 21, 23).

Immediately following inoculation the organisms were located in the outer surface of the membrane (Plate 24). Within two days they had penetrated the inner surface of the membrane and made contact with the limiting membrane. The number of bacteria appeared to have increased evenly throughout the membrane. There was no evidence that micro-organisms had accumulated against the limiting membrane (Plate 24). Of the bacteria that were observed in contact with the latter, only one appeared to be associated with a point of degradation (Plate 27). Likewise bacteria interacting with the fibre mantles were a rare occurrence. Thus this study did not provide evidence in support of the suggestion (Candlish, 1972) that extensive degradation of the membrane fibre is a consequence of bacterial contamination.

Although, as was demonstrated (Fig. 23), the number of cfu increased in the membrane during the "storage" period, there was no detectable contamination of the albumen over this period. However, within 24 hours of raising the incubation temperature to 37.5°C the detectable level of albumen contamination had risen by over seven log cycles. There was no evidence that the sudden increase in the level of detectable contaminants was connected with a breakdown of the limiting membrane.

Once organisms have migrated from the shell membranes into the albumen they will be subjected to a broad spectrum of antimicrobial agents (Tranter and Board, 1982a). The agents' action may be modified by many factors. For instance incubating the fertile (or infertile) egg may bring about changes in the albumen's antimicrobial properties eg a change in albumen pH that occurs naturally post-oviposition. The effects that egg incubation and

the change in albumen pH might have on the growth of salmonellae in albumen were therefore studied.

SALMONELLA SP. - GROWTH IN ALBUMEN

Species and inoculum size

The following species of salmonellae were incubated separately (37°C/shaking water bath) in bulked albumen: S. hadar, S. virchow, S. anatum, S. goelitz and S. typhimurium.

There were certain notable differences between the species in terms of their ability to survive in hens' egg albumen. As might be anticipated from the previous studies (Table 5/7, Fig. 21/23), S. hadar (Fig. 24g) was more resistant - the number of cfu increasing as incubation proceeded - to the inimical environment of the albumen than the other species examined. While the number of S. typhimurium (Fig. 24c) cfu remained static throughout the incubation periods, the viable counts for the other species declined as incubation progressed. The numbers of S. goelitz (Fig. 24d), for instance, remained static for the first three hours of incubation, declined by ca. one log unit over the subsequent three hours and then remained static for the remainder of the incubation period. Salmonella anatum (Fig. 24e) on the other hand did not manifest an initial static phase, the number of cfu declining by more than three log units during the course of incubation. As in the membrane contamination study (Fig. 20), the growth curves for a given species at the different inoculum levels tended to run parallel to each other.

Albumen pH

Following oviposition CO_2 diffuses out of the albumen causing the pH to increase from ca. 7.4 to 9.6. The albumen's antimicrobial

properties are known to be enhanced (Tranter and Board, 1982a) at the ultimately high alkaline pH that results. Carbon dioxide is also lost from albumen transferred to flasks. The rate and extent of the change in the pH of albumen incubated in an Erlenmyer flask (shaking water bath/37.5°) is shown in Fig. 25. Thus when incubating albumen which has a relatively low pH, it must be appreciated that, depending on various factors (eg temperature, CO_2 gradient), the pH is liable to increase as incubation progresses.

The effect of an increase in albumen pH by less than one pH unit is clearly demonstrated by Fig. 24a/c and b/d. At the lower pH (8.62) the cfu of S. typhimurium and S. goelitz both increased - although the response was not as marked at the lower (10^4 cfu/membrane) inoculum level. At the higher pH level the populations were either static (S. typhimurium/Fig. 24c) or diminished (S. goelitz/Fig. 24d) - only the cfu of the S. hadar population increased significantly. Other results shown in Table 9 support these observations. Indeed similar findings were reported by Banwart and Ayres (1957) who found that the cfu of S. pullorum, S. oranenburg and S. senftenberg declined when these organisms were inoculated into egg albumen adjusted to pH nine or ten.

It is interesting to note that whereas the growth curves for a given organism at the different inoculation levels tended to run parallel to each other at the higher pH level (Fig. 24c, d), there was a tendency for the curves to diverge at the lower pH values (Fig. 24a, b).

Change in the albumen's antimicrobial efficacy during incubation

The eggs used for this section of the study were stored for three days (16°C/r.h. 75%) - conditions that are often used

(Jones, 1981) for the storage of hatching eggs - by which time the albumen pH had stabilised at ca. 9.6.

When organisms were inoculated into egg albumen taken from eggs on Day 0 of incubation (Fig. 26/27 and Table 10/11), the number of cfu remained static (24 h/37°C) there being no detectable difference in the albumen taken from fertile or infertile eggs. Indeed there was no notable difference at any stage of the incubation between the antimicrobial efficacy of the albumen taken from fertile or infertile eggs. Unfortunately only a limited number of fertile eggs could be examined because the problems associated with aseptically removing albumen from fertilised eggs after more than a few days incubation are considerable due to the development of the embryonic membranes and the weakening of the yolk membrane. By the third day of incubation the number of S. hadar (Fig. 26) cfu had increased by half a log cycle over the 24 h incubation period (S. typhimurium increased by 0.3 of a log cycle over the same period). Albumen from eggs that had been incubated for ten days was associated with the number of S. hadar and S. typhimurium cfu (Fig. 26/27) to increasing by >1.5 log units during the 24 h incubation period. Moreover it was found that after ten days of incubation, further incubation had no discernible influence on the antimicrobial properties of the albumen, suggesting that under these conditions (37.5°C/r.h. 60%) maximum deterioration in the albumen's efficacy had occurred by the tenth day of incubation.

Decreasing the inoculum level by ca. two log units to 10^6 cfu/ml albumen (Fig. 28/29 and Table 12/13) caused an overall decline in cfu - although the albumens' antimicrobial efficacy was again most effective during the first few days of incubation, the efficacy decreasing as incubation proceeded. It was notable that

although there was no significant difference in the growth curves of S. hadar and S. typhimurium at the higher inoculum level, S. hadar appeared more resistant to the albumens' inimical environment than S. typhimurium at the lower inoculum level. For instance at the lower inoculum level the number of S. typhimurium cfu (day 0) decreased over the 24 h incubation period by ca. three log cycles (cf S. hadar, < two log cycles). Moreover by day 21 of incubation the number of S. typhimurium cfu had decreased by only one log unit over the 24 h incubation period, the corresponding number of S. hadar cfu was nearly static. For reasons that at present are unknown, S. hadar appears to be better adapted than the other salmonellae examined in this study to survive and grow in the inimical environment of the hens' egg albumen.

In summary the pattern of growth in albumen of all the Salmonella spp. examined was dependant on a number of factors. Of these probably the most important was inoculum size, as it determined the rate and extent of detectable albumen contamination. As might be expected albumen pH was shown to be important, the initial low pH favouring growth. Throughout this section of the study there were indications that S. hadar was more resistant to the albumen's unfavourable environment than the other species examined.

The effect of albumen incubation temperature on the growth of salmonellae

The experiments described above were concerned with the development of microbial contaminants within the integument and albumen of the incubating egg. The bacteria were therefore incubated at 37.5°C. The effect of other, pertinent, incubation temperatures was studied also in order to provide a better understanding of the

growth of microbial contaminants in egg albumen. The temperatures were chosen to include those obtained in refrigerated storage, ambient storage and at the body temperature of the hen. The albumen's pH prior to incubation was ca. 9.6.

Generally the bactericidal properties of the albumen were enhanced as the incubation temperature increased (Fig. 30). As already noted, the Salmonella spp. were not affected equally by the albumen's hostile environment. For instance the number of S. kedogan (Fig. 30b) cfu diminished from ca. 10^5 to $<10^2$ cfu/ml within three hours at an incubation temperature of 44°C, although the number of S. hadar (Fig. 30d) cfu required up to 7.5 hours to reach the same level. As incubation temperature diminished so the bactericidal quality of the albumen diminished also until at ca. 25 - 30°C for all but one of the species examined (S. kedogan) it was only bacteriostatic. With an incubation temperature of 6.5°C the viable populations remained almost static. The results of other experiments, which also showed these trends, are shown in Table 14.

It is interesting to note that biologically the toxicity of the albumen is nearly maximal at around "natural" incubation temperature. This suggests that natural selection has favoured the evolution of an egg albumen having a chemical defence that operates maximally at this temperature.

TABLE 3. Factors associated with the egg integument that may promote water uptake.

1) Lack of cuticle (ie scratched or cuticle-less)

2) Cracked shell

3) Oversized or "patent" pores

4) Calcareous lumps

TABLE 4. Bacteria isolated from faecal inoculum.

Family	Genus	
Enterbacteriaceae	I	Escherichia coli type 1
	III	Citrobacter freundii
	III	Citrobacter diversus/amalonaticus
	IV	Salmonella sp.
	VI	Klebsiella pneumoniae
	VII	Enterobacter sakazakii
	VII	Enterobacter cloacae
	X	Proteus vulgaris
	X	Providencia rettgeri*
	X	Providencia alcalifaciens*
Neisseriaceae	IV	Acinetobacter calco var. lwoffi
Coryneform	IV	Kurthia zopfii**
	IV	Kurthia gibsonia**

* Proteus inconstans has been given generic rank as Providencia by Ewing (1958, 1962).

** Identified according to Shaw and Keddie (1983).

TABLE 5. The effect of inoculum level on the viable counts obtained from hen eggs at 37.5°C/r.h. 60% inoculated in the air cell with faecal material.

Number (Log_{10}) cfu/membrane

		Time (d)		
		0	2	4
Total count	(1)	5.89*	>9.00	>9.00
	(2)	4.69	7.11	8.04
	(3)	3.68	5.45	7.51
	(4)	2.39	3.53	4.21
	(5)	<2.00	<2.00	<2.00
Coliforms	(1)	5.55*	>9.00	>9.00
	(2)	4.62	7.00	7.98
	(3)	3.60	5.41	7.25
	(4)	2.00	<2.00	<2.00
	(5)	<2.00	< 2.00	< 2.00
Enterococci	(1)	3.42*	4.90	6.00
	(2)	2.31	4.00	3.12
	(3)	<2.00	<2.00	<2.00
	(4)	<2.00	<2.00	<2.00
	(5)	<2.00	< 2.00	<2.00

TABLE 5. (cont)

Number (Log_{10}) cfu/membrane

		Time (d)		
		0	2	4
Salmonella hadar	(1)	3.21*	5.98	6.62
	(2)	2.60	5.35	6.01
	(3)	2.00	4.30	5.60
	(4)	<2.00	<2.00	<2.00
	(5)	<2.00	<2.00	<2.00
Micrococci	(1)	2.90*	<2.00	<2.00
	(2)	2.00	<2.00	<2.00
	(3)	<2.00	<2.00	<2.00
	(4)	<2.00	<2.00	<2.00
	(5)	<2.00	<2.00	<2.00

Number (Log_{10}) cfu/ml of albumen

Total count	(1)	<2.00+	2.62	5.98
	(2)	<2.00	2.00	2.71
	(3)	<2.00	<2.00	<2.00
	(4)	<2.00	<2.00	<2.00
	(5)	<2.00	<2.00	<2.00

* Mean of eight membranes

\+ Mean of eight eggs

TABLE 8. Development of faecal contaminants inoculated into the air cell of incubated (37.5°C/r.h. 60%) infertile eggs.

(a) Number (Log_{10}) cfu/membrane

	Time (d)						
	0	2	4	8	12	14	19
Total count	3.11*	4.98	5.21	5.00	3.41	7.60	8.54
Coliforms	2.96	<2.00	<2.00	<2.00	<2.00	7.54	8.12
Pseudomonads	2.82	<2.00	<2.00	<2.00	<2.00	7.30	8.08
Enterococci	<2.00	<2.00	<2.00	<2.00	<2.00	2.81	3.20
Micrococci	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
Salmonella virchow	<2.00	<2.12	2.00	<2.00	<2.00	2.40	2.98

Number (Log_{10}) cfu/albumen

Total count	<2.00+	<2.00	<2.00	<2.00	<2.00	9.84	10.82

* Mean of eight membranes

\+ Mean of eight eggs

TABLE 6. (cont)

(b) Number (Log_{10}) cfu/membrane

	Time (d)						
	0	2	4	8	12	14	19
Total count	2.90*	5.10	4.10	3.60	3.10	7.97	9.20
Coliforms	2.85	<2.00	<2.00	<2.00	<2.00	7.62	7.50
Pseudomonads	<2.00	<2.00	<2.00	<2.00	<2.00	7.58	7.80
Enterococci	<2.00	<2.00	<2.00	<2.00	<2.00	2.21	4.12
Micrococci	<2.00	<2.00	<2.00	<2.00	<2.00	2.41	<2.00
Salmonella anatum	<2.00	<2.00	2.24	<2.00	<2.00	2.61	3.12

Number (Log_{10}) cfu/ml albumen

Total count	<2.00+	<2.00	<2.00	<2.00	<2.00	10.41	10.31

* Mean of eight membranes

\+ Mean of eight eggs

TABLE 7. Development of faecal contaminants inoculated into the air cell of incubated (37.5°C/r.h. 60%) infertile eggs

(a) Number (Log_{10}) cfu/membrane

	Time (d)						
	0	2	4	8	11	14	19
Total count	6.24*	8.10	7.83	8.63	9.07	9.85	9.90
Coliforms	6.12	6.26	7.00	7.12	8.52	9.10	9.32
Pseudomonads	3.56	2.12	<2.00	<2.00	<2.00	6.32	6.63
Enterococci	3.58	4.62	3.02	2.96	3.59	4.71	5.12
Micrococci	2.51	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
Salmonella virchow	3.25	3.50	3.75	4.48	5.71	5.75	5.52

Number (Log_{10}) cfu/ml albumen

Total count	<2.00+	<2.00	<2.00	<2.00	<2.00	9.68	9.85

* Mean of eight membranes + Mean of eight eggs

TABLE 7. (cont)

(b) Number (Log_{10}) cfu/membrane

	Time (d)						
	0	2	4	8	11	14	19
Total count	5.54*	7.71	8.12	8.98	9.38	10.08	9.89
Coliforms	5.26	6.98	7.31	6.80	8.50	8.75	8.89
Pseudomonads	4.00	<2.00	<2.00	<2.00	<2.00	5.81	6.25
Enterococci	2.96	3.96	3.42	3.54	3.46	4.32	4.87
Micrococci	<2.00	2.42	2.31	<2.00	<2.00	<2.00	<2.00
Salmonella hadar	2.31	2.91	3.59	4.61	5.00	5.80	5.98

* Mean of eight membranes

+ Mean of eight eggs

Number (Log_{10}) cfu/ml albumen

	Time (d)						
Total count	<2.00+	<2.00	<2.00	<2.00	<2.00	8.54	9.67

TABLE 8. The effect of "storage" (16°C/r.h. 75%) and subsequent incubation (37.5°C/r.h. 60%) on faecal contaminants inoculated into the air cell.

Number (Log_{10}) cfu/membrane

	Time (d)			
	0	7**	9	13
Total count	3.11*	5.02	8.12	7.75
Coliforms	2.96	4.97	8.00	7.60
Pseudomonads	2.82	4.89	7.97	7.32
Enterococci	<2.00	<2.00	2.97	<2.00
Micrococci	<2.00	<2.00	2.31	<2.00
Salmonella virchow	<2.00	<2.00	3.12	3.06

Number (Log_{10}) cfu/ml albumen

Total count	<2.00+	<2.00	8.87	9.24

* Mean of eight membranes

** End of "storage"

\+ Mean of eight eggs

TABLE 9. The effect of pH on Salmonellae (inoculum-dependent) growth in hens' egg albumen.

pH 8.54 Number (Log_{10}) cfu/ml albumen

	Time (h)							
	0	1.5	3.0	4.5	6.0	7.5	9.0	24
S. typhimurium	5.50	6.00	6.50	7.03	7.10	7.51	7.75	7.90
	4.75	5.02	5.25	5.51	5.65	5.50	5.63	5.71
	3.51	3.89	4.00	4.03	4.45	4.52	4.50	4.48
S. goelitz	5.21	5.92	6.56	6.82	6.80	7.51	8.04	8.34
	4.00	4.28	4.45	4.50	4.32	4.68	4.62	4.91
	2.50	2.52	2.49	2.63	2.47	2.55	2.46	2.02
S. hadar	5.25	5.56	6.02	6.20	6.35	6.82	7.22	7.04
	4.10	4.23	4.28	4.75	5.00	5.03	5.35	5.50
	3.00	3.10	3.51	4.01	4.10	4.49	4.51	4.11
pH 9.61								
S. typhimurium	4.53	4.57	4.75	4.70	4.63	4.65	4.49	4.84
	3.82	3.51	3.50	3.65	3.61	3.68	3.64	3.91
	3.10	2.88	3.00	2.91	2.52	2.43	2.35	2.56
S. goelitz	4.95	4.84	4.53	4.36	4.03	3.78	3.68	3.42
	3.81	3.79	3.48	3.08	2.76	2.51	2.48	2.54
	3.15	3.00	2.12	2.00	2.00	2.00	2.00	2.00

TABLE 9. (cont)

pH 9.61 Number (Log_{10}) cfu/ml of albumen

	Time (h)							
	0	1.5	3.0	4.5	6.0	7.5	9.0	24
S. hadar	5.00	5.31	5.61	5.75	5.91	6.03	6.10	6.45
	4.65	4.00	4.26	4.48	4.73	4.81	4.80	4.71
	2.81	2.95	3.02	2.89	2.81	2.76	2.59	2.50

TABLE 10. The effect of egg incubation (37.5°C/r.h. 60%) on the albumen's antimicrobial efficacy.

Salmonella hadar — Number (Log_{10}) cfu/ml albumen

Age of albumen (days)	Time (h) 0	1.5	3.0	4.5	6.0	7.5	9.0	24.0
0	7.71* (7.60)+	7.60 (7.57)	7.55 (7.50)	7.61 (7.63)	7.65 (7.68)	7.60 (7.65)	7.58 (7.60)	7.50 (7.55)
3	7.65 (7.68)	7.89 (7.75)	8.10 (8.08)	8.18 (8.20)	8.21 (8.12)	8.25 (8.20)	8.23 (8.08)	8.00 (7.88)
6	7.70 (7.65)	7.80 (7.85)	8.25 (8.10)	8.50 (8.43)	8.72 (8.48)	8.85 (8.78)	8.94 (8.89)	9.12 (9.02)
10	7.61	8.13	8.70	8.95	9.08	9.15	9.21	9.06
18	7.63	8.21	8.95	9.12	9.25	9.38	9.41	9.30
21	7.83	8.35	8.98	9.18	9.26	9.34	9.30	9.13

* Mean of three samples - taken from infertile eggs.

\+ Mean of three samples - taken from fertile eggs.

TABLE 11. The effect of egg incubation (37.5°C/r.h. 60%) on the albumen's antimicrobial efficacy.

Salmonella typhimurium — Number (Log_{10}) cfu/ml albumen

Age of albumen (days)	Time (h) 0	1.5	3.0	4.5	6.0	7.5	9.0	24.0
0	7.80* (7.71)+	7.82 (7.75)	7.73 (7.70)	7.85 (7.65)	7.79 (7.71)	7.83 (7.76)	7.71 (7.81)	7.65 (7.73)
3	7.71 (7.83)	7.95 (7.85)	8.00 (7.93)	8.08 (7.81)	7.95 (8.00)	7.82 (7.92)	7.91 (7.85)	7.89 (7.99)
6	7.85 (7.81)	8.18 (8.12)	8.22 (8.14)	8.28 (8.14)	8.13 (8.21)	8.39 (8.37)	8.56 (8.50)	8.53 (8.64)
10	7.83	8.42	8.72	9.00	9.09	9.20	9.32	9.30
18	7.75	8.61	8.93	9.10	9.22	9.32	9.35	9.34
20	7.81	8.50	8.89	9.18	9.29	9.41	9.42	9.39

* Mean of three samples - taken from infertile eggs.

\+ Mean of three samples - taken from fertile eggs.

TABLE 12. The effect of egg incubation (37.5°C/r.h. 60%) on the albumen's antimicrobial efficacy.

Salmonella hadar — Number (Log_{10}) cfu/ml albumen

Age of albumen (days)	Time (h) 0	1.5	3.0	4.5	6.0	7.5	9.0	24
0	6.01* (5.89)+	5.75 (5.69)	5.40 (5.36)	4.91 (4.87)	4.59 (4.58)	4.36 (4.41)	4.18 (4.10)	3.08 (3.00)
3	5.95 (5.95)	5.81 (5.74)	5.75 (5.87)	5.20 (5.36)	4.81 (4.93)	4.45 (4.60)	4.21 (4.31)	3.88 (4.00)
6	5.86 (6.09)	5.91 (6.00)	5.90 (5.97)	5.61 (5.68)	5.08 (5.70)	4.62 (4.62)	4.43 (4.58)	4.58 (4.42)
10	6.08	6.10	6.00	5.85	5.70	5.43	5.22	4.71
18	6.00	5.80	6.00	5.97	5.88	5.80	5.58	5.36
21	5.96	6.00	6.10	6.05	6.00	5.98	5.91	5.82

* Mean of three samples - taken from infertile eggs.

\+ Mean of three samples - taken from fertile eggs.

TABLE 13. The effect of egg incubation (37.5°C/r.h. 60%) on the albumen's antimicrobial efficacy.

Salmonella typhimurium — Number (Log_{10}) cfu/ml albumen

Age of albumen (days)	Time (h)											
0	5.81* (5.73)+	5.75 (5.70)	5.31 (5.11)	4.86 (5.03)	4.26 (4.31)	3.38 (3.35)	2.51 (2.41)	2.00 (2.00)
3	5.96 (5.74)	5.79 (6.02)	5.50 (5.69)	4.88 (4.89)	4.54 (4.66)	4.02 (4.08)	3.21 (3.30)	2.98 (3.12)
6	5.75	5.90	5.60	5.31	5.08	4.62	4.03	3.89
10	5.83	5.88	5.71	5.46	5.25	5.09	4.41	4.21
18	5.71	5.83	5.73	5.52	5.25	5.00	4.46	3.82
21	5.92	5.86	5.75	5.63	5.51	5.23	4.97	4.13

* Mean of three samples - taken from infertile eggs.

\+ Mean of three samples - taken from fertile eggs.

TABLE 14. The effect of incubation temperature on the toxicity of hens' egg albumen.

Salmonella anatum — Number (Log_{10}) cfu/ml albumen

Temperature (°C)	Time (h) 0	1.5	3.0	4.5	6.0	7.5	9.0	10.5	24
44.0	5.00	4.20	3.12	2.25	<2.00	<2.00	<2.00	<2.00	<2.00
39.5	5.10	4.91	4.85	3.88	<2.00	<2.00	<2.00	<2.00	<2.00
30.0	4.92	5.18	5.10	4.95	4.81	4.69	4.45	4.37	4.35
25.0	4.96	5.25	5.31	5.28	5.20	5.15	5.10	5.06	4.89
6.5	5.12	5.08	5.25	5.21	5.01	5.05	5.00	5.05	5.09

Salmonella kedogan

Temperature (°C)									
44.0	5.10	3.32	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
39.5	4.95	4.50	3.89	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
30.0	4.90	4.89	4.75	4.75	4.45	4.21	4.18	4.08	3.10
25.0	4.98	5.00	5.30	5.26	5.18	5.05	5.15	5.10	5.20
6.5	5.00	5.12	5.20	5.14	5.20	5.18	5.03	5.10	5.15

TABLE 14. (cont)

Salmonella virchow

Number (Log_{10}) cfu/ml albumen

Temperature (°C)	Time (h) 0	1.5	3.0	4.5	6.0	7.5	9.0	10.5	24
44.0	5.10	4.10	2.61	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
39.5	4.86	4.81	3.56	2.81	<2.00	<2.00	<2.00	<2.00	<2.00
30.0	4.98	5.21	5.03	4.85	4.32	4.02	4.23	4.18	4.10
25.0	4.91	5.15	5.08	5.10	4.98	4.75	4.82	4.93	4.51
6.5	5.00	5.10	4.91	5.03	4.81	4.85	4.97	5.02	4.86

Salmonella hadar

Temperature (°C)									
44.0	4.92	4.45	3.78	3.43	2.85	<2.00	<2.00	<2.00	<2.00
39.5	5.03	4.92	4.53	4.25	4.01	3.67	3.79	3.68	<2.00
30.0	5.10	5.13	5.16	4.87	4.75	4.77	4.62	4.45	3.93
25.0	4.96	5.21	5.18	5.13	5.15	5.20	5.08	5.12	4.86
6.5	4.90	5.12	5.18	5.38	5.25	5.39	5.32	5.35	5.28

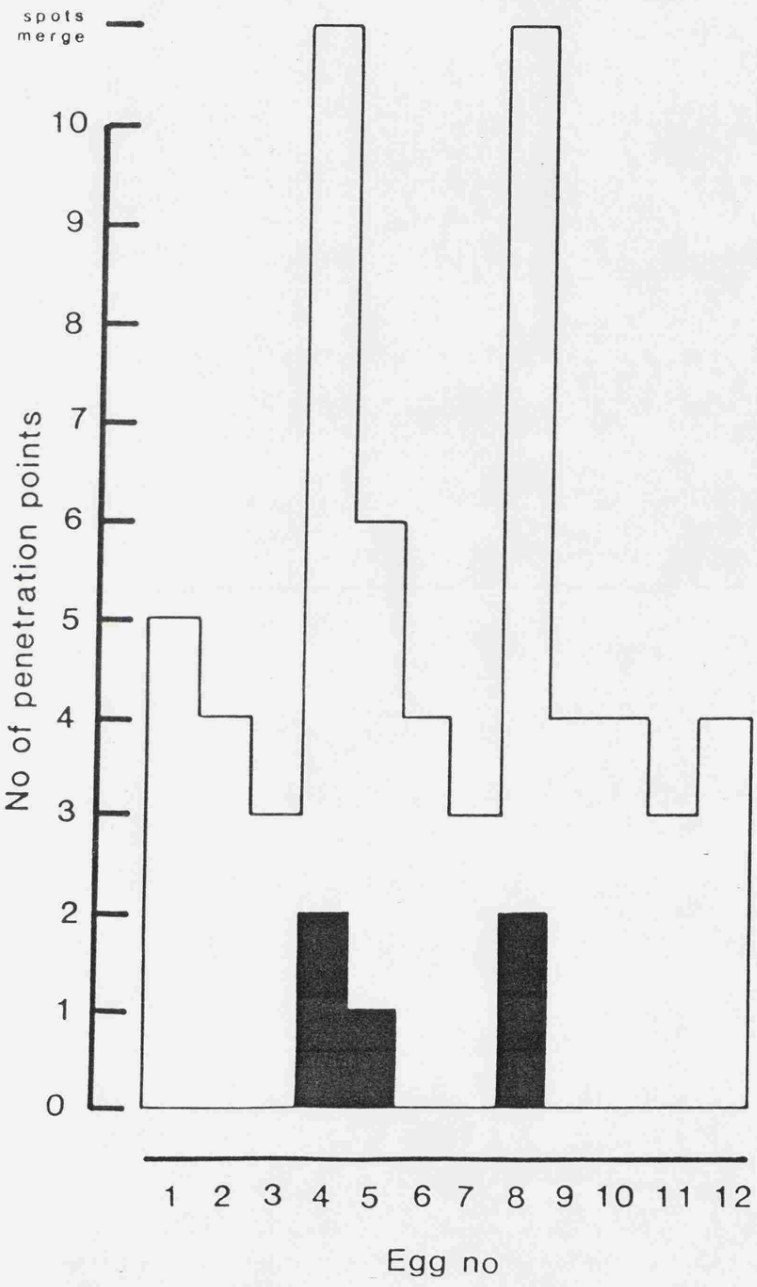
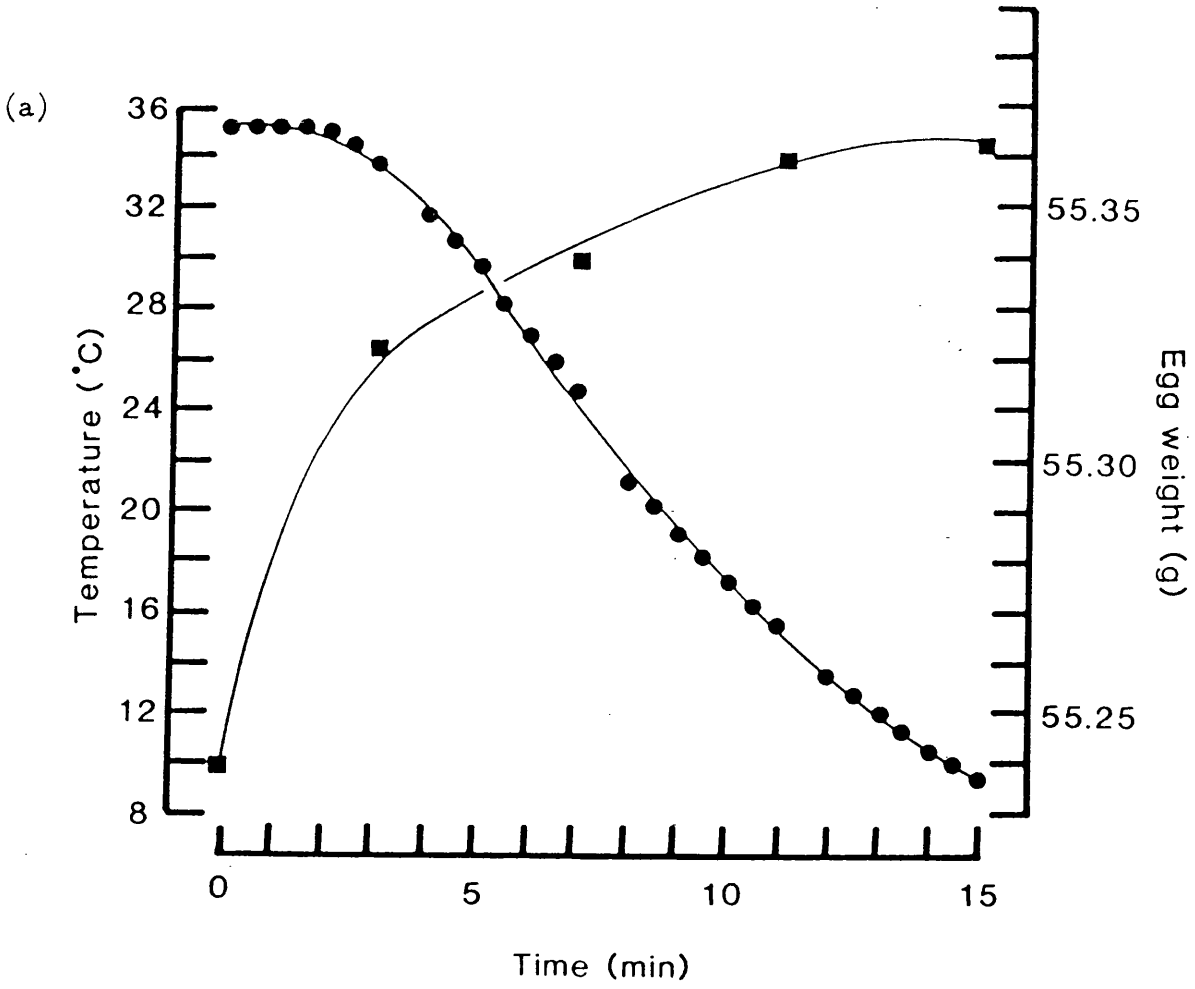


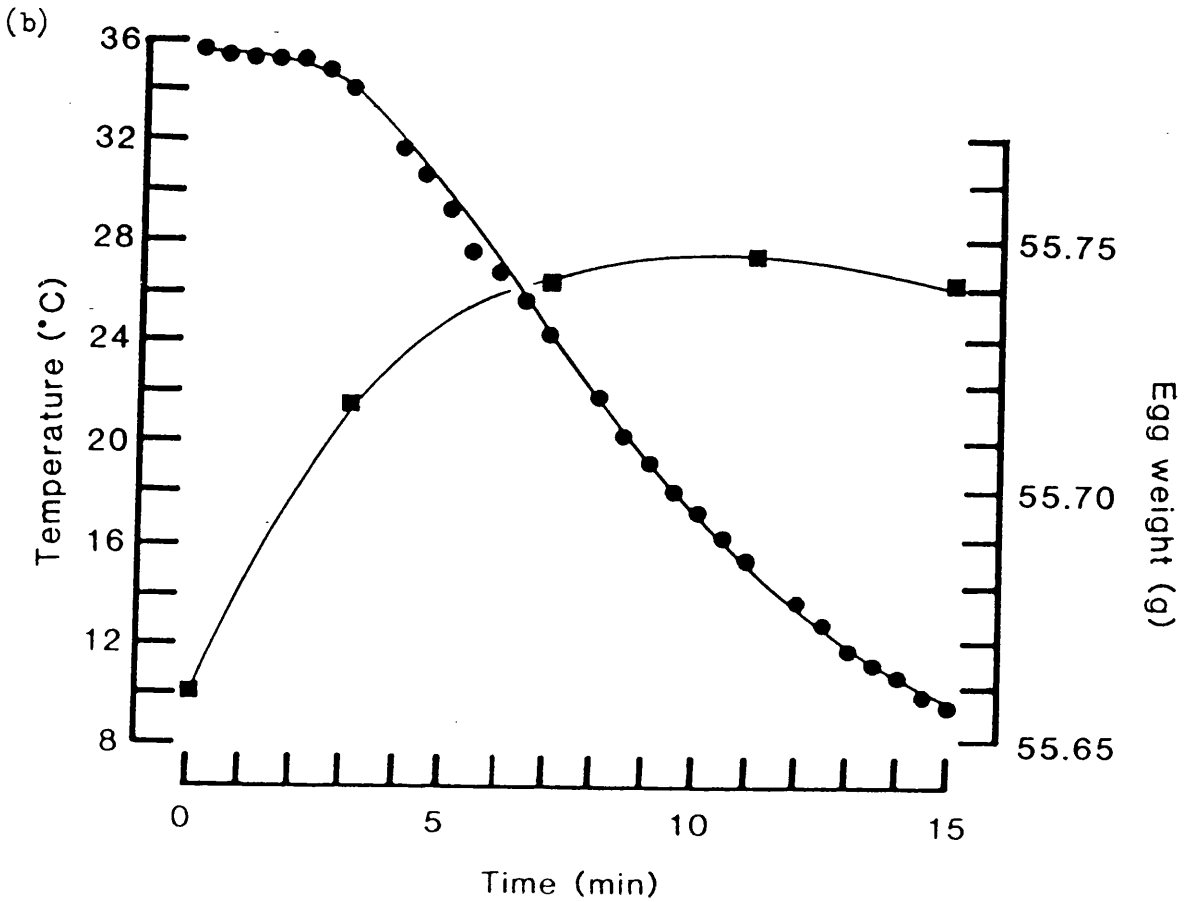
Fig. 8 The efficacy of mature (solid columns) and immature (open columns) cuticles in preventing the penetration of faecal bacteria through the egg integument. Penetration points are the result of the microbial reduction of 2, 3, 5-triphenyltetrazolium chloride to formazan.

Fig. 9 The rate of decrease (caused by plunging warm eggs - 36°C - into slush ice - 4°C) in core temperature (closed circle) of eggs varying in weight from 55.24g (a) to 59.50g (d) was identical ($p < 0.001$). Increase in egg weight caused by water uptake (closed squares).

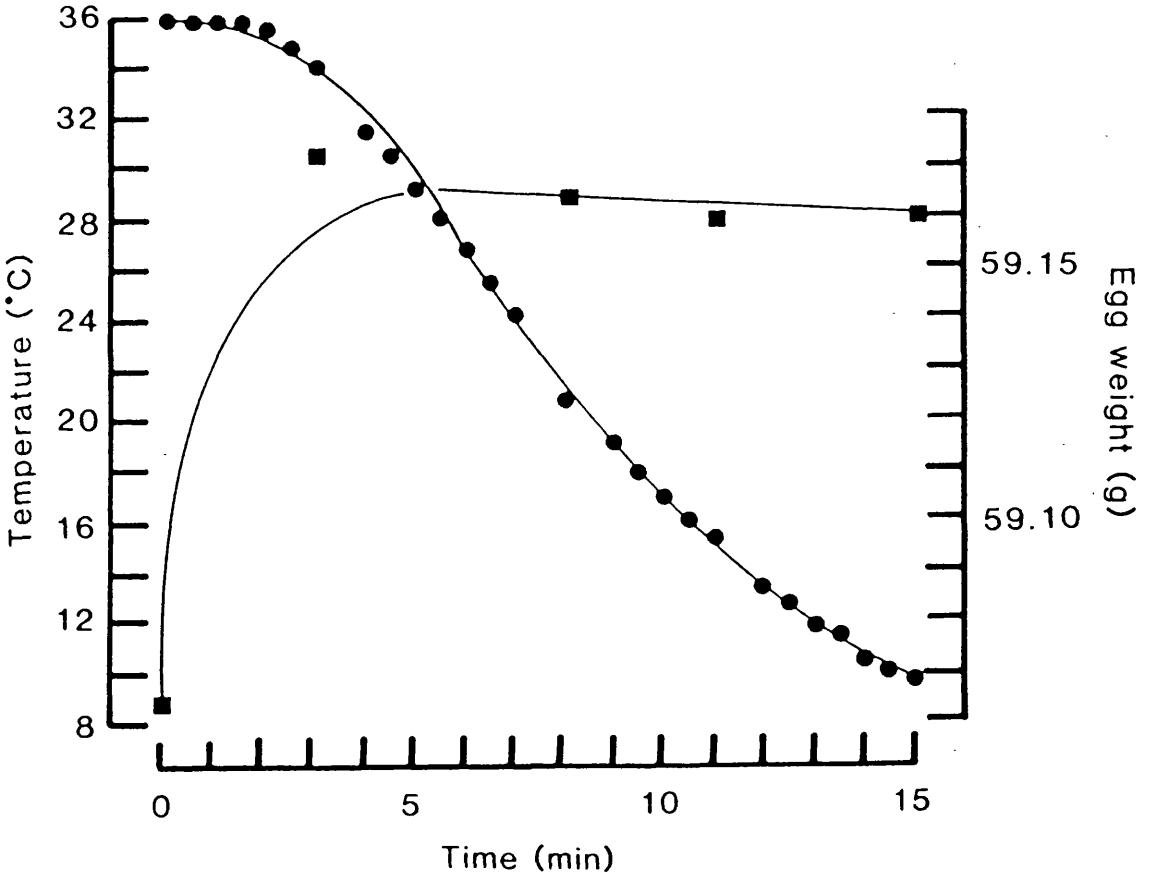
Fig. 9

180

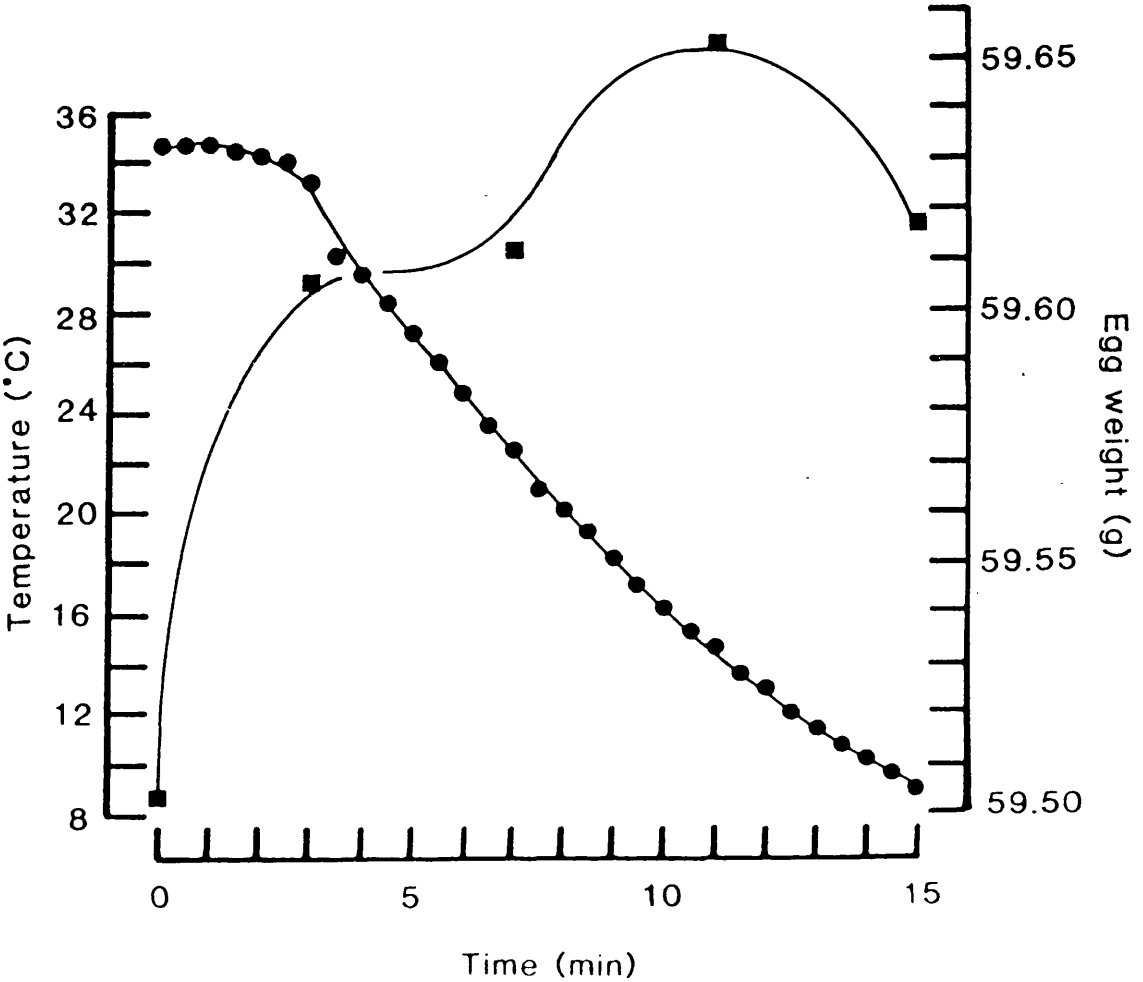

(a)
36
32
28
24
20
16
12
8
Temperature (°C)
55.35
55.30
55.25
Egg weight (g)
0
5
10
15
Time (min)


(b)
36
32
28
24
20
16
12
8
Temperature (°C)
55.75
55.70
55.65
Egg weight (g)
0
5
10
15
Time (min)

(c)


36
32
28
24
20
16
12
8
Temperature (°C)
59.15
59.10
Egg weight (g)
0
5
10
15
Time (min)

(d)


36
32
28
24
20
16
12
8
Temperature (°C)
59.65
59.60
59.55
59.50
Egg weight (g)
0
5
10
15
Time (min)

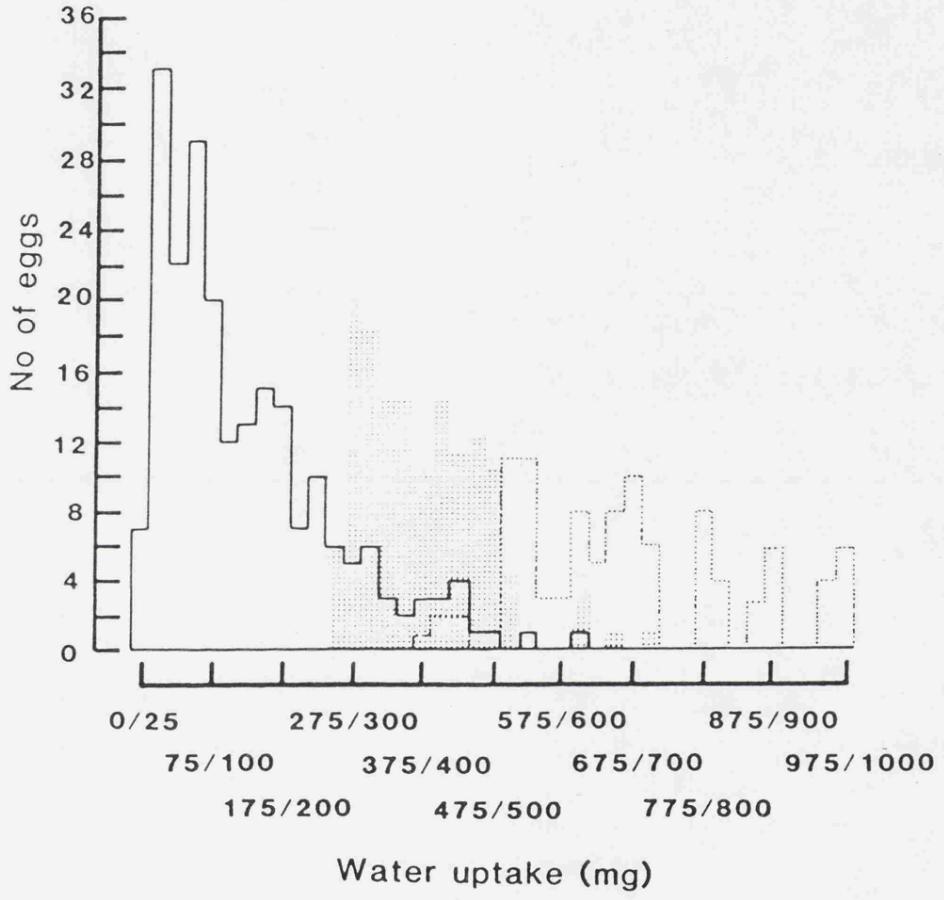


Fig. 10 Water uptake (induced by a temperature differential dip) was found to be greater in white eggs (stippled column) than brown eggs (open column). Brown eggs which had been stored (open column, broken border) for three days (37°C/r.h. 0%) took up an even greater amount due to the effect of the enlarged air cell.

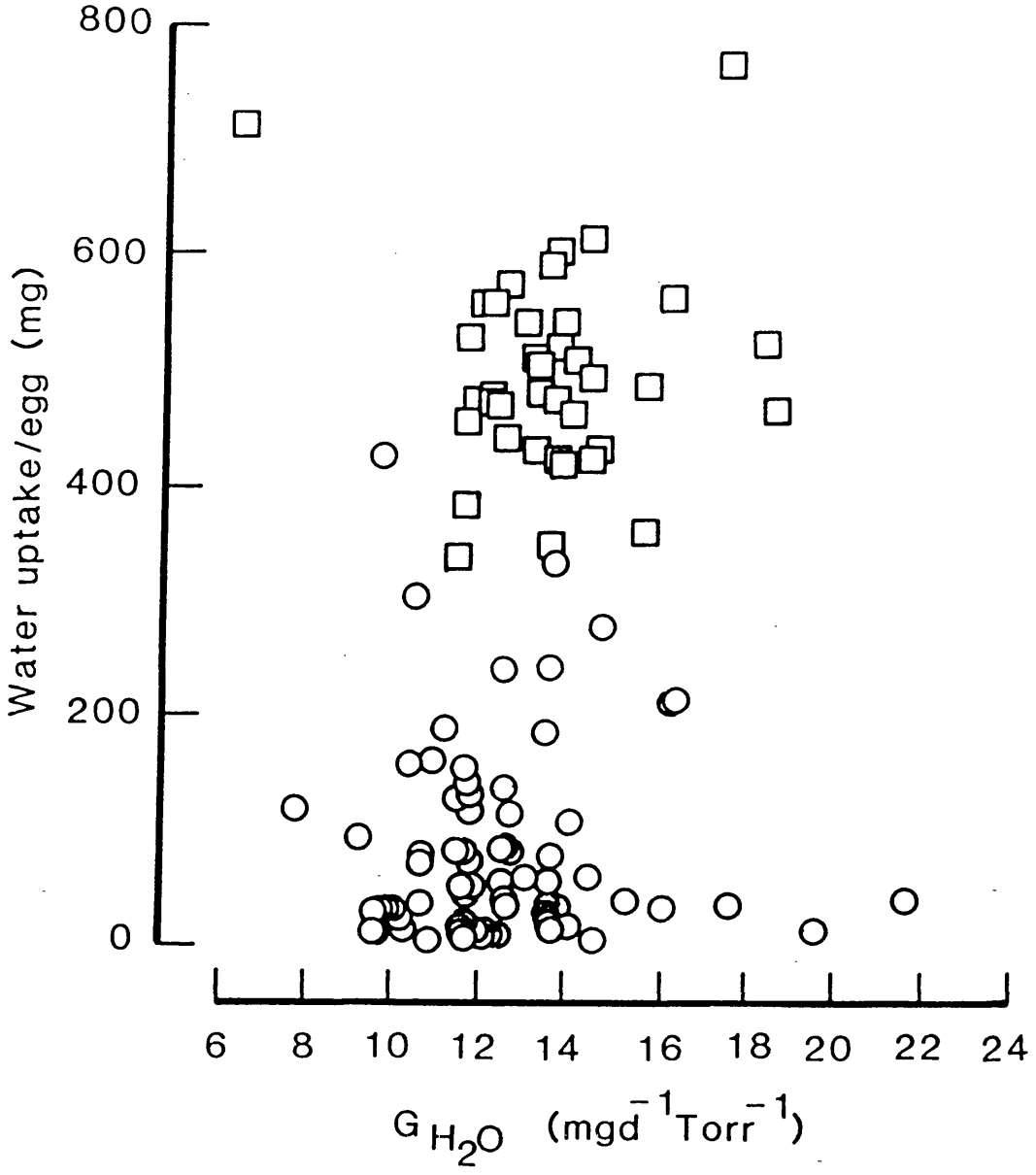


Fig. 11 Removal of the cuticle (open square) increases water uptake compared with untreated controls (open circle) but does not affect G_{H_2O} values.

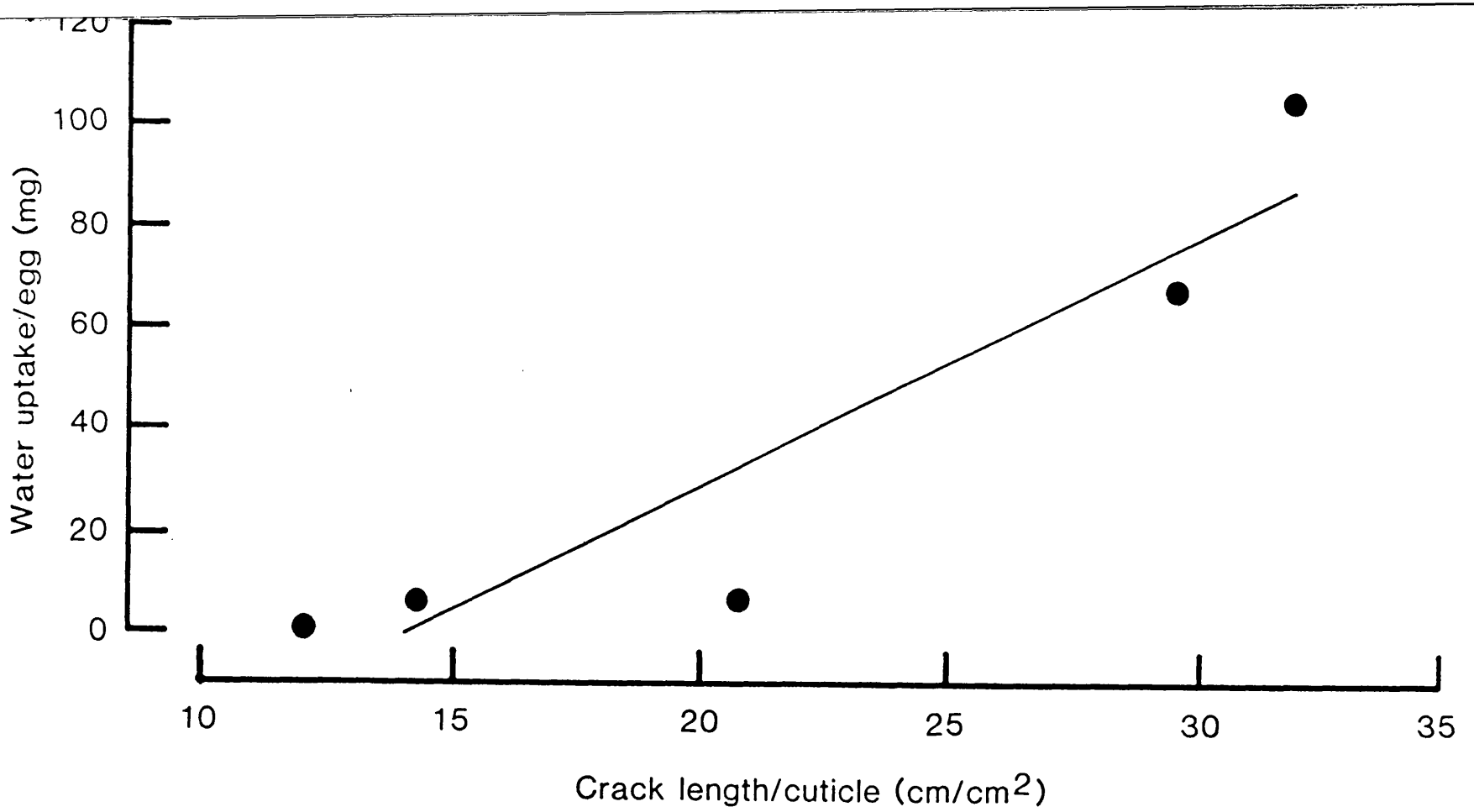
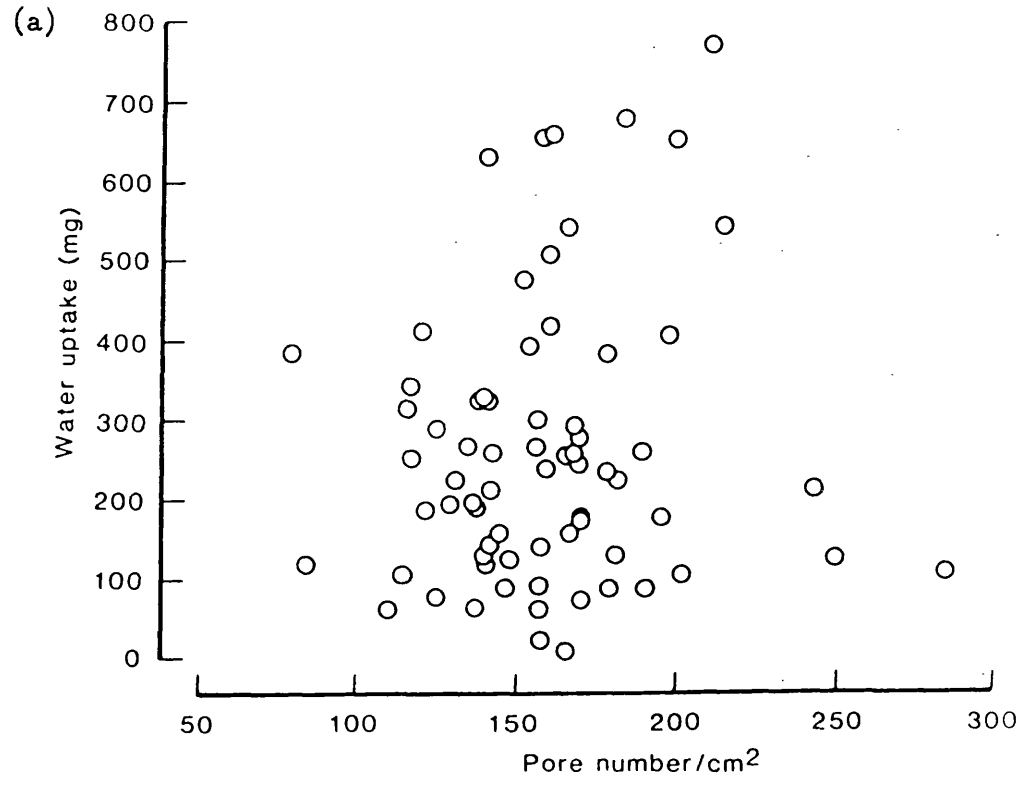


Fig. 12 There was a significant linear correlation ($r = 0.93$, $p<0.05$) between the degree of fissuring in the cuticle of five eggs, taken from high, medium and low water uptake groups.

Fig. 13 There was no correlation between water uptake and pore number (a) or G_{H_2O} (b). Each point represents one egg.

Fig. 13


(a)
800
700
600
500
400
300
200
100
0
Water uptake (mg)
50
100
150
200
250
300
Pore number/cm2

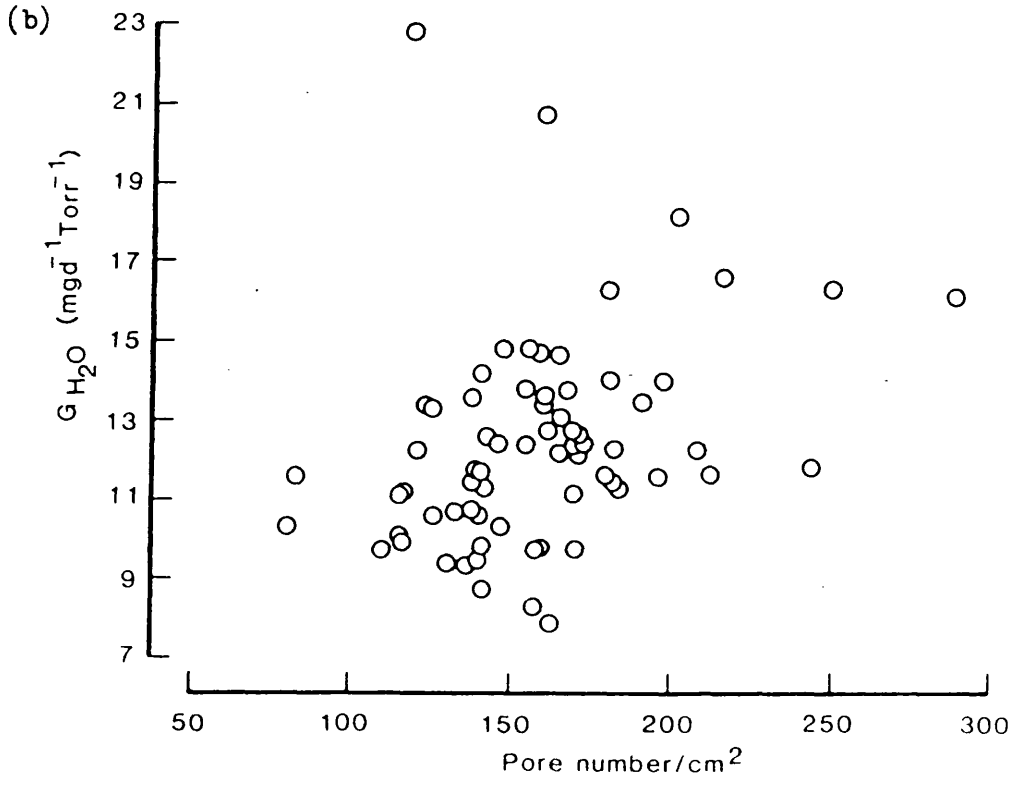

(b)
23
21
19
17
15
13
11
9
7
G H2O (mgd-1Torr-1)
50
100
150
200
250
300
Pore number/cm2

Fig. 14 The G_{H_2O} values (open column) of consecutive eggs tended to increase and then decrease at the beginning and end respectively of each clutch. No trend was apparent for pore number (solid column). Incomplete clutches are denoted by a ragged boundary.

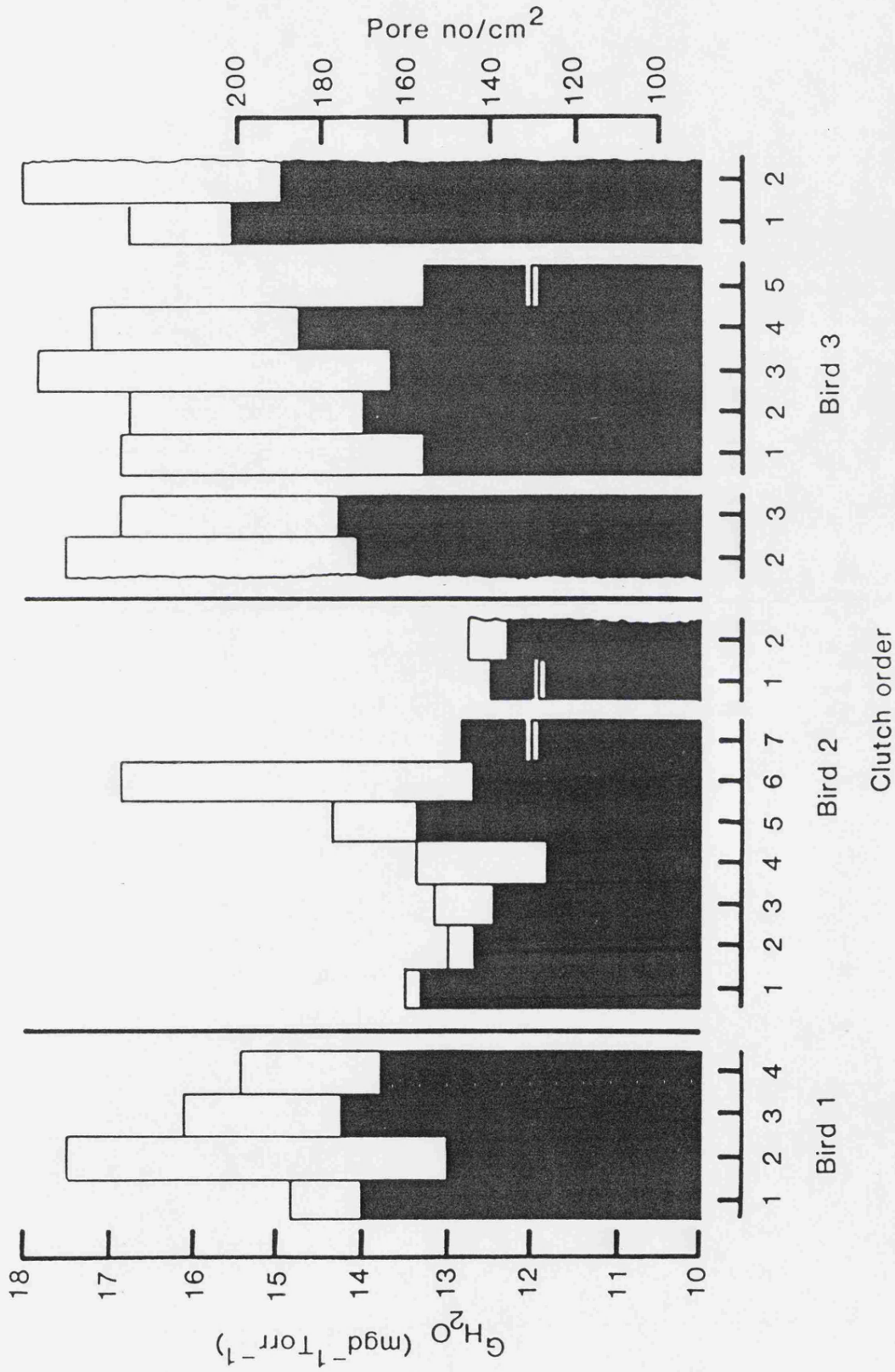

Pore no/cm^2
200
180
160
140
120
100
G_{H_2O} (mgd^{-1}Torr^{-1})
18
17
16
15
14
13
12
11
10
1 2 3 4
1 2 3 4 5 6 7
1 2
2 3
1 2 3 4 5
1 2
Bird 1
Bird 2
Bird 3
Clutch order

Fig. 14

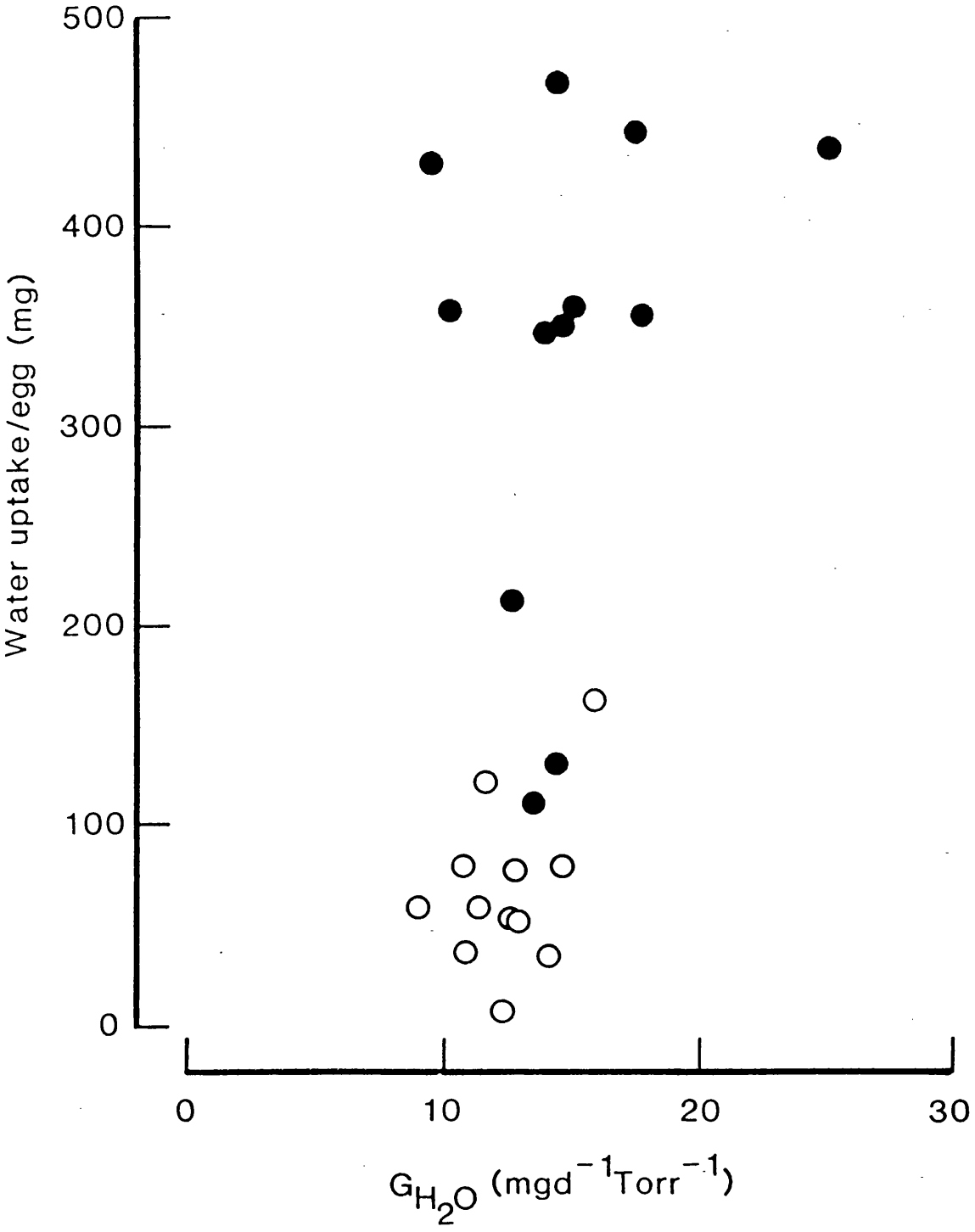


Fig. 15 Adding a surfactant (Triton X-100, closed circles) to the differential dip solution caused a dramatic increase in water uptake (brown eggs) compared with the controls (open circles).

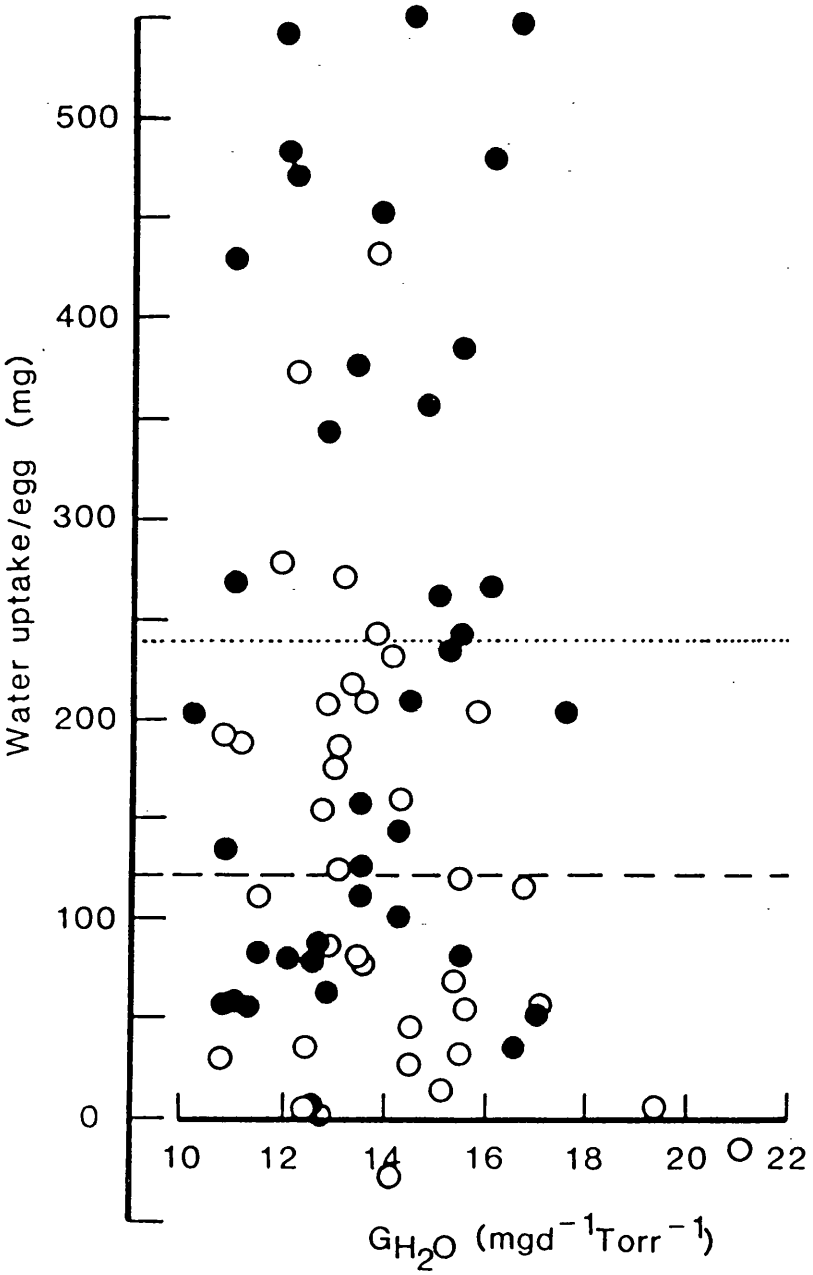
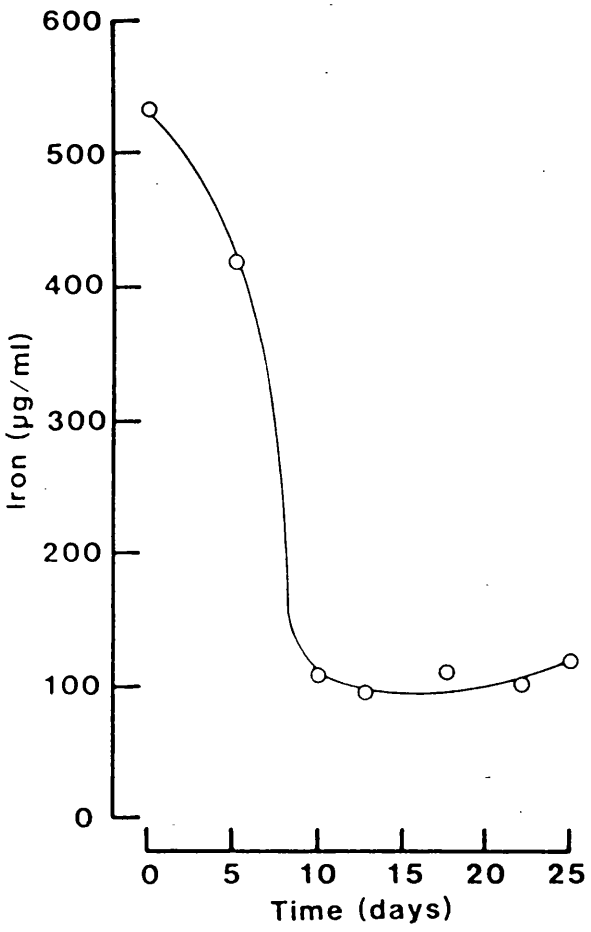


Fig. 16 Degassing the temperature differential dip solution (closed circle) increased the mean water uptake value (••••••) by over 100mg compared with the mean (------) of the controls (open circle).



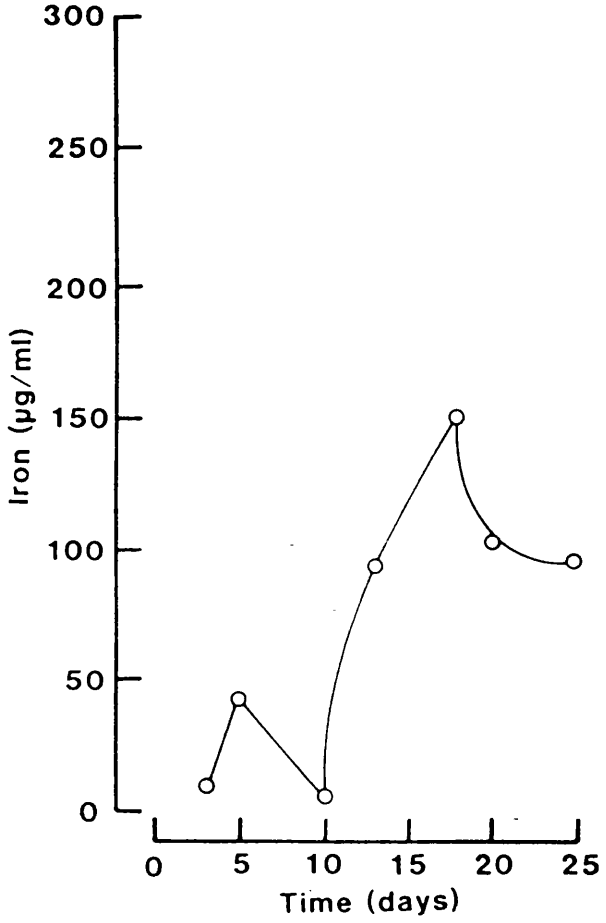


Fig. 17

(a) Change in the concentration of iron (0.1 ml of 6 mg $Fe(NH_4)_2(SO_4)_2.6H_2O$/ml water) placed on the inner shell membrane of incubating (37.5°C/r.h. 60%) infertile eggs. Each point represents the mean of 3 eggs.

(b) Change in the concentration of iron in the albumen taken from those eggs examined in Fig. 17(a).

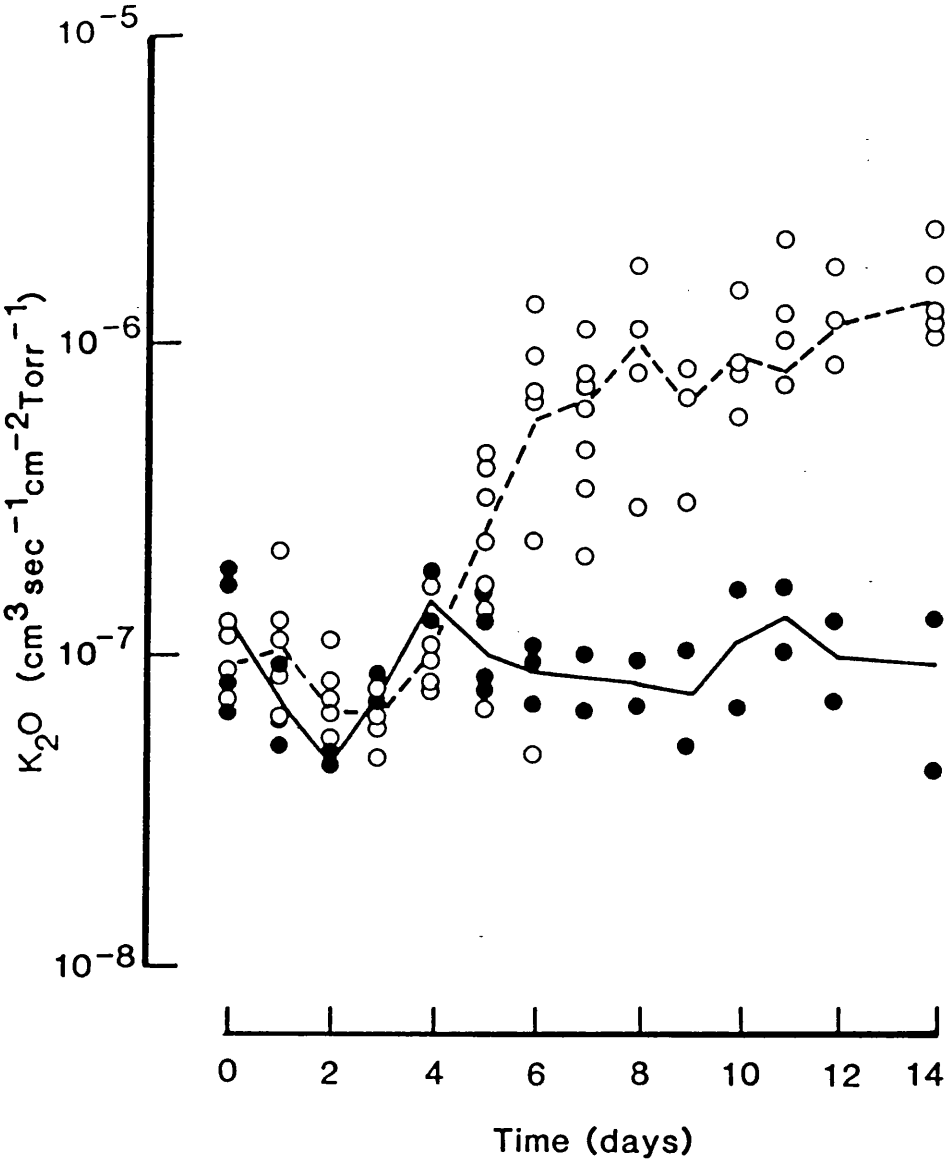


Fig. 18 The rate of oxygen transfer across the shell and shell membranes of infertile (closed circles and solid line - mean) and fertile (open circles and broken line - mean) eggs of the domestic hen at different times during incubation. Each symbol is the result obtained from one egg incubated (37.5°C/r.h. 60%) with turning for the period shown.

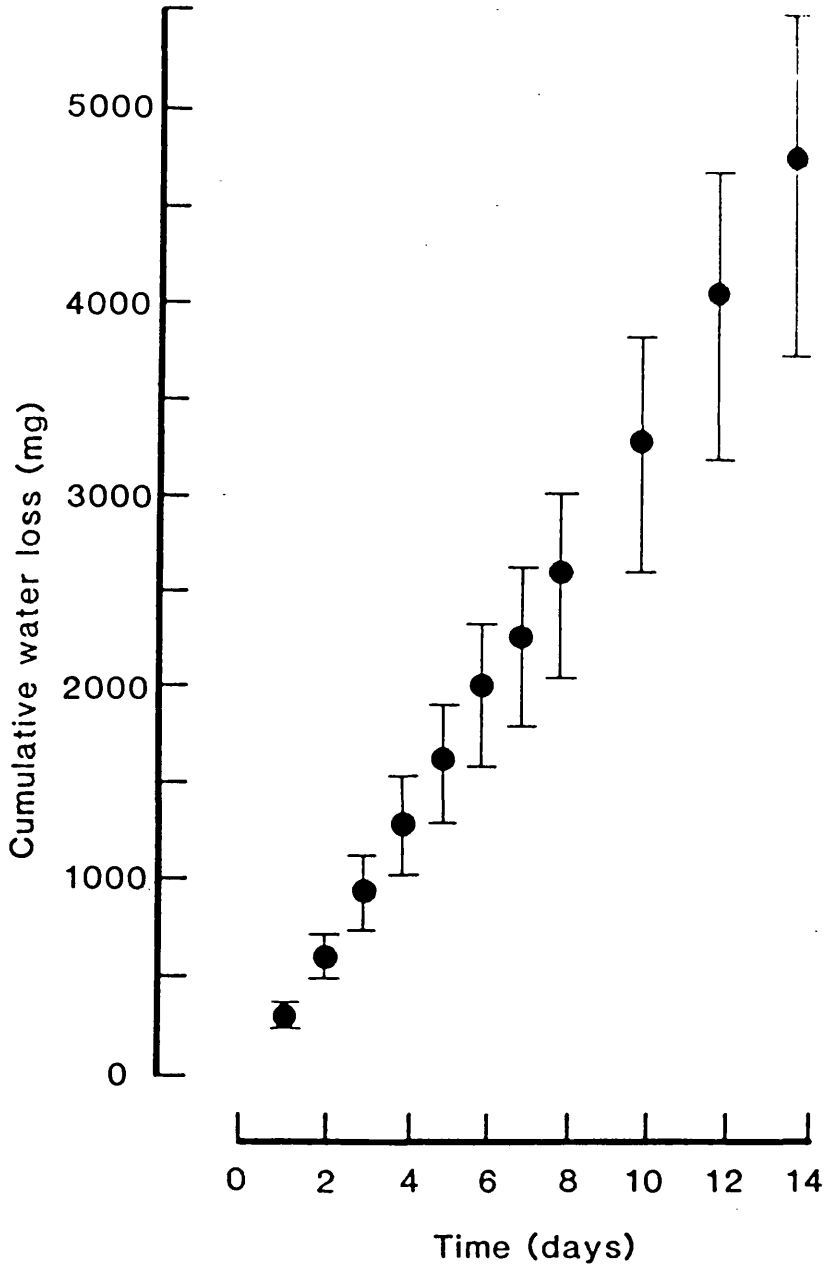


Fig. 19 The weight loss from 12 fertile eggs of the domestic hen during incubation (37.5°C/r.h. 60%) with turning. Closed circle - mean, bars - range.

Fig. 20

a) The effect of inoculum (hen faeces) level on the growth of organisms (total count) inoculated onto the inner shell membrane of incubated eggs (37.5°C/r.h. 60%). Each point represents the mean of eight membranes.

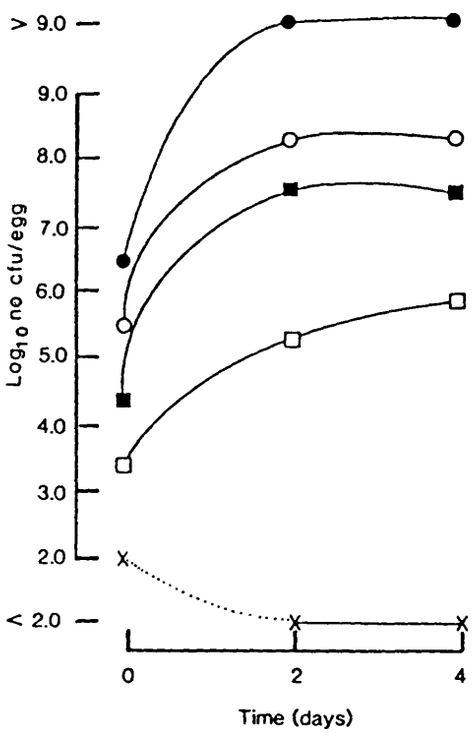
b) The effect of inoculum level on the growth of coliform organisms (originating from the faecal inoculum, Fig. 20a). Details as for Fig. 20a.

c) The effect of inoculum level on the growth of enterococci (originating from the faecal inoculum, Fig. 20a). Details as for Fig. 20a.

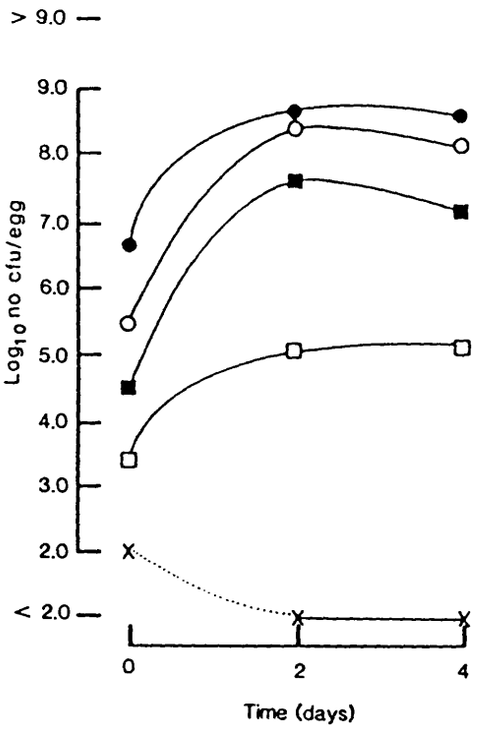
d) The effect of inoculum level on the growth of Salmonella anatum (added to the faecal inoculum, Fig. 20a). Details as for Fig. 20a.

Fig. 20

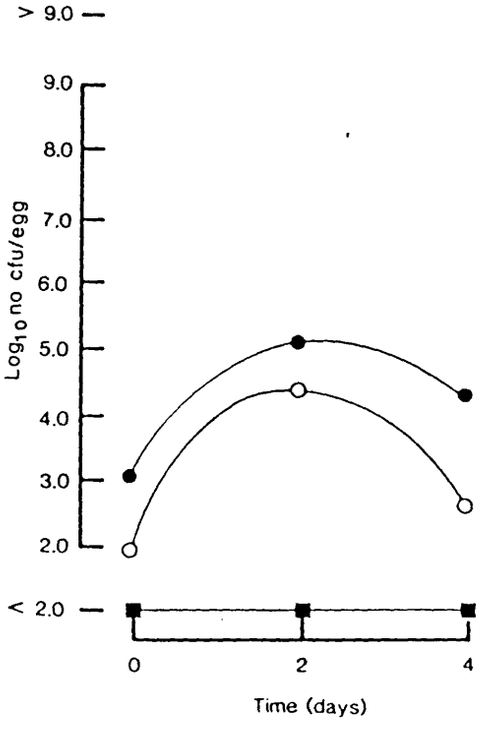
a)


> 9.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
< 2.0
Log10 no cfu/egg
0
2
4
Time (days)

b)


> 9.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
< 2.0
Log10 no cfu/egg
0
2
4
Time (days)

)


> 9.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
< 2.0
Log10 no cfu/egg
0
2
4
Time (days)

d)

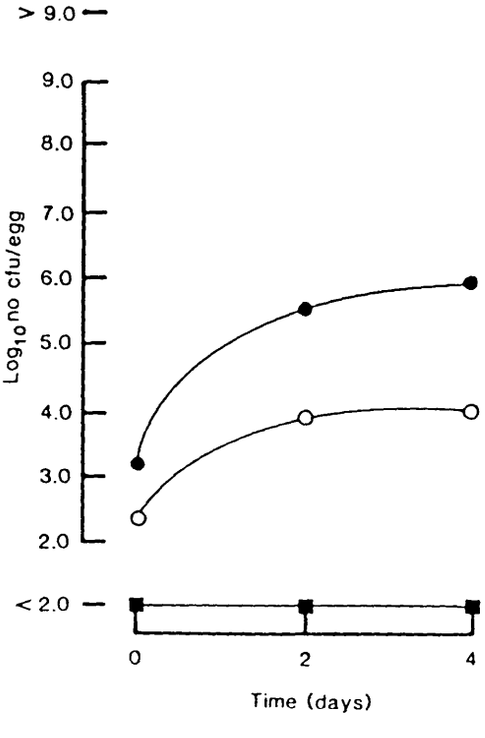

> 9.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
< 2.0
Log10 no cfu/egg
0
2
4
Time (days)

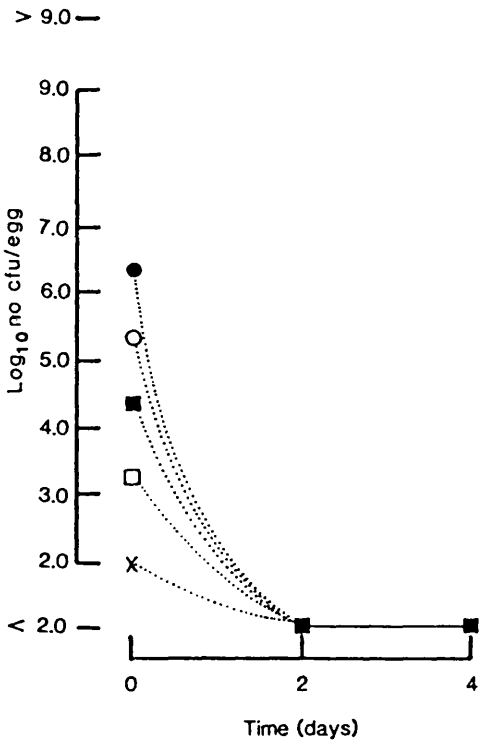
Fig. 20 (cont)

e) The effect of inoculum level on the growth of micrococci (originating from the faecal inoculum, Fig. 20a). Details as for Fig. 20a.

f) The effect of inoculum level (Fig. 20a) on the extent of contamination (total count) of the albumen. Details as for Fig. 20a.

Fig. 20 (cont)

e)



f)

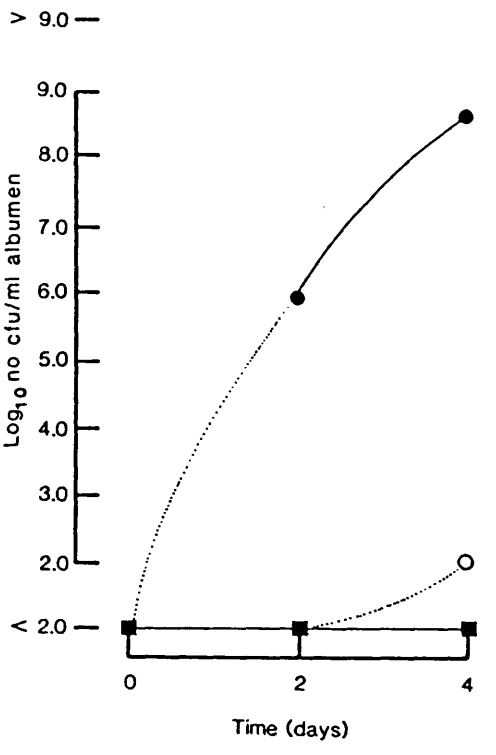


Fig. 21

a) The effect of extended egg incubation (37.5°C/r.h. 60%) on the growth of organisms inoculated onto the inner shell membrane. Each point represents the mean of eight membranes.

b) The effect of extended egg incubation (37.5°C/r.h. 60%) on the growth of coliforms (originating from the faecal inoculum, Fig. 21a). Details as for Fig. 21a.

c) The effect of extended egg incubation (37.5°C/r.h. 60%) on the growth of enterococci (originating from the faecal inoculum, Fig. 21a). Details as for Fig. 21a.

d) The effect of extended egg incubation (37.5°C/r.h. 60%) on the growth of pseudomonads (originating from the faecal inoculum, Fig. 21a). Details as for Fig. 21a.

Fig. 21

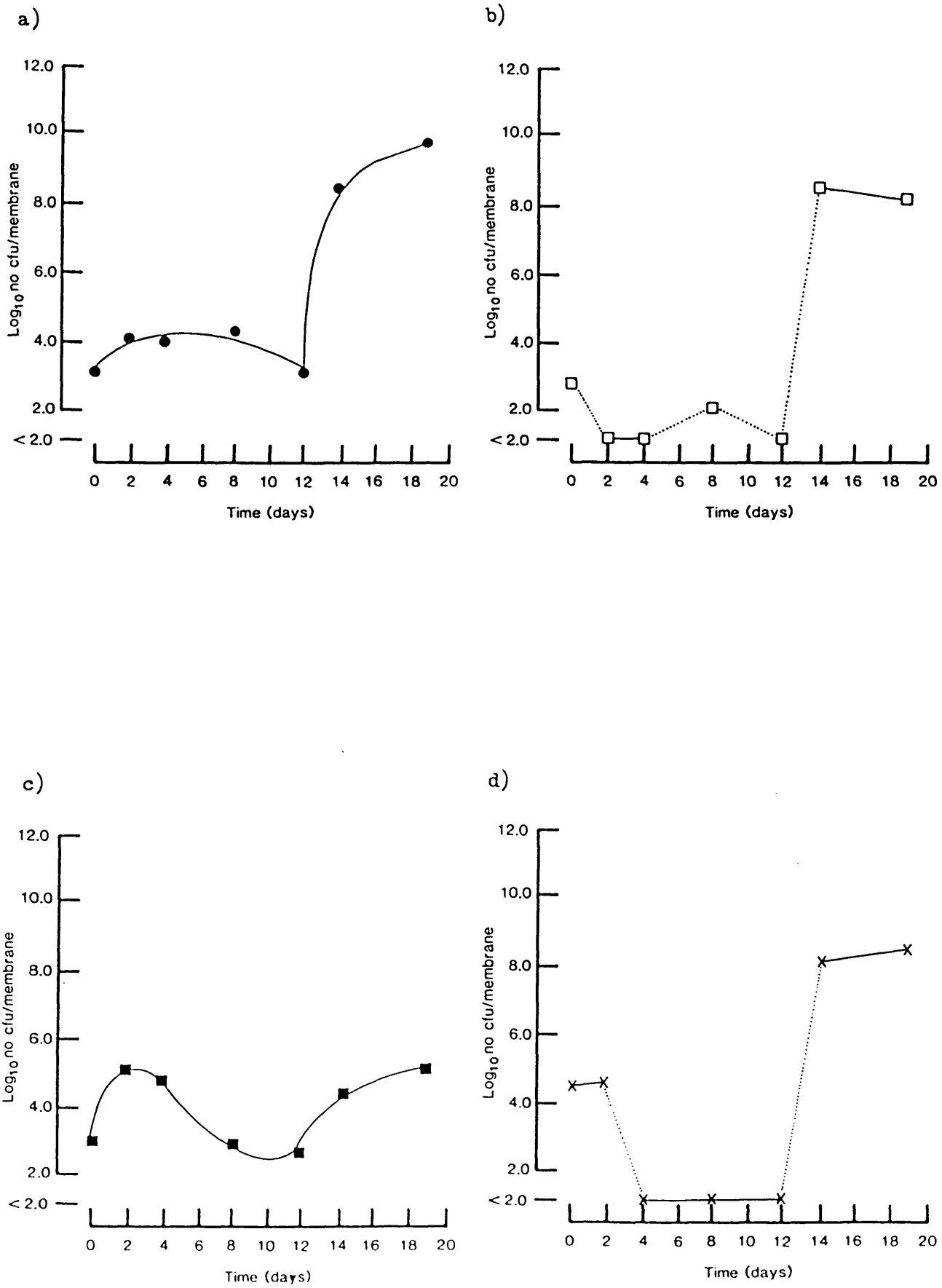

a)
b)
c)
d)
12.0
10.0
8.0
6.0
4.0
2.0
< 2.0
Log10 no cfu/membrane
0 2 4 6 8 10 12 14 16 18 20
Time (days)

Fig. 21 (cont)

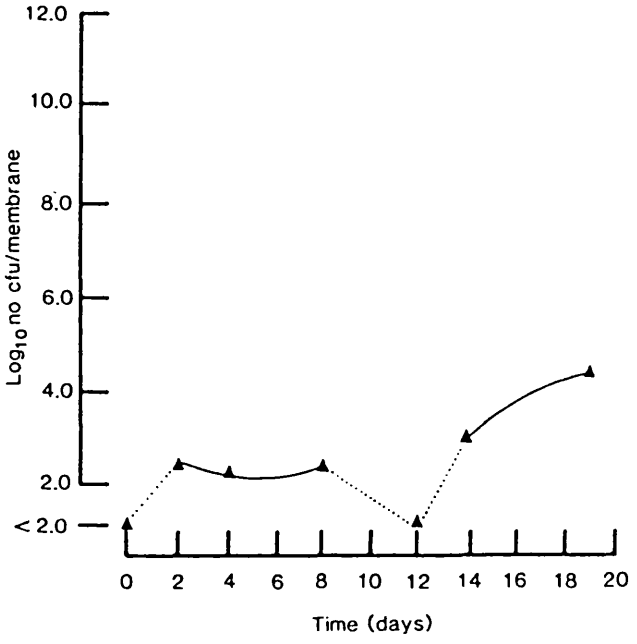
e) The effect of extended egg incubation (37.5°C/r.h. 60%) on the growth of Salmonella hadar (added to the faecal inoculum, Fig. 21a). Details as for Fig. 21a.

f) The effect of extended egg incubation (37.5°C/ r.h. 60%) on the growth of micrococci (originating from the faecal inoculum, Fig. 21a). Details as for Fig. 21a.

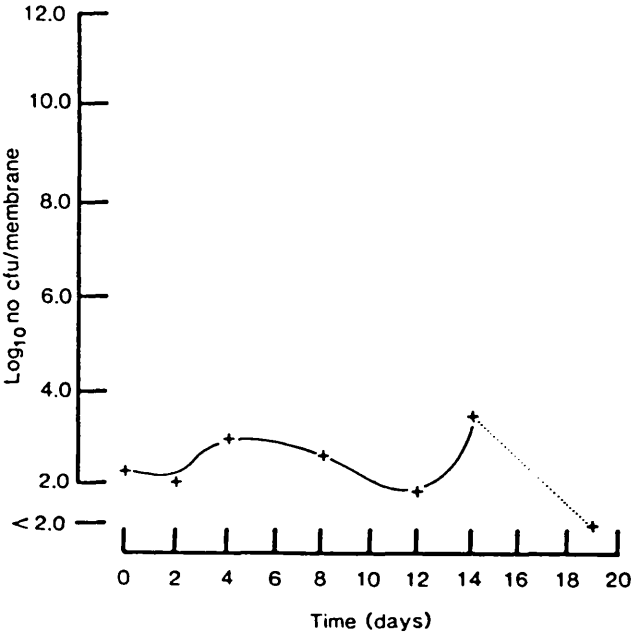
g) The effect of extended egg incubation (37.5°C/r.h. 60%) on the extent of contamination (total count) of the albumen. Details as for Fig. 21a.

Fig. 21 (cont)

e)



f)



g)

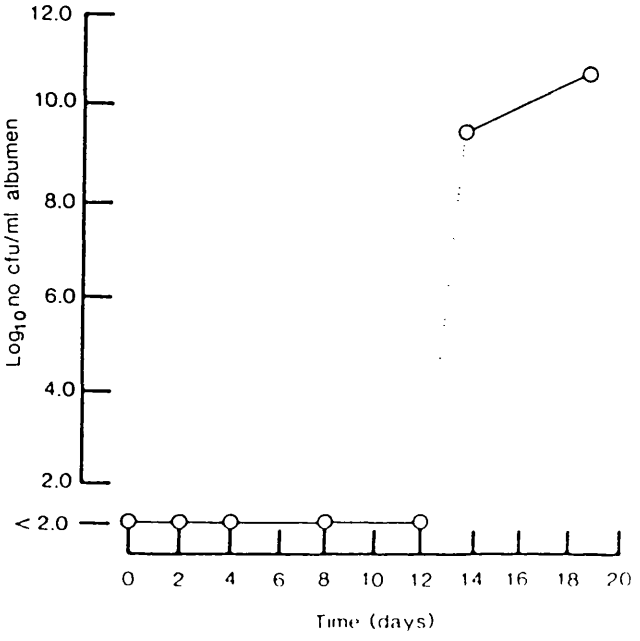


Fig. 22

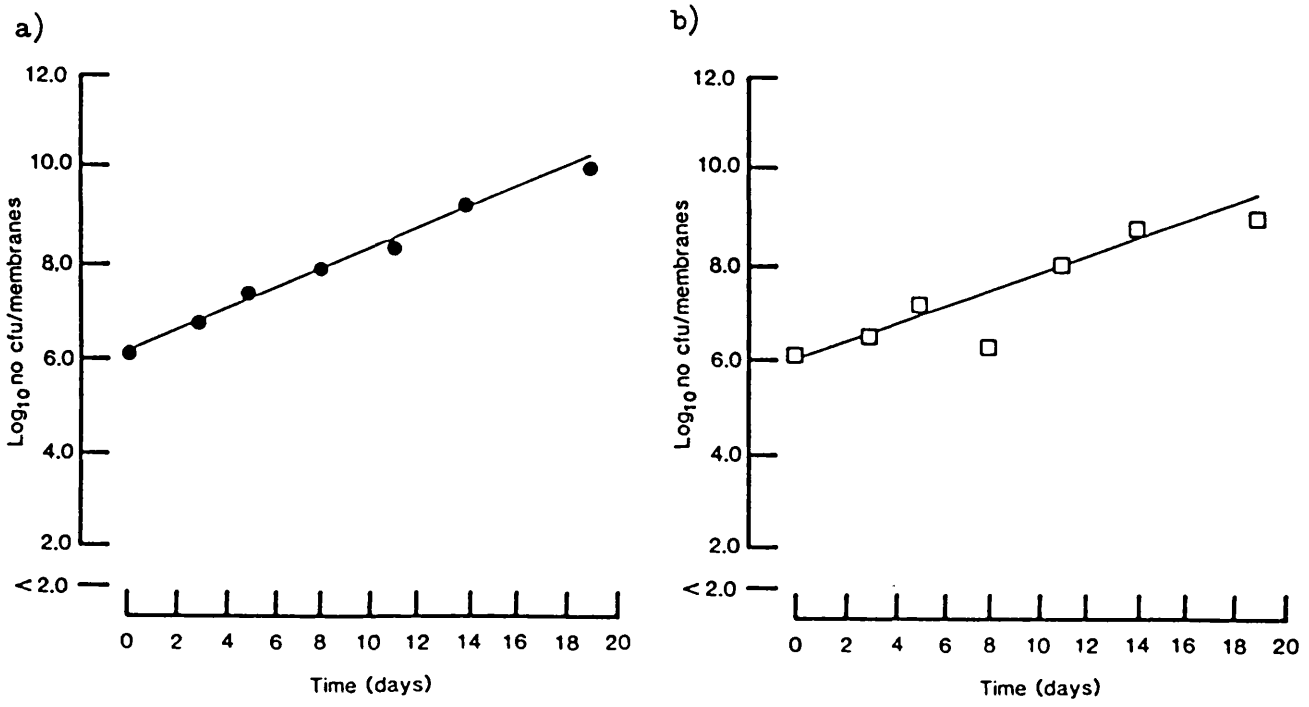
a) The effect of extended egg incubation (37.5°C/r.h. 60%) on the growth of organisms inoculated onto the inner shell membrane. Note the effect of the increased inoculum level cf Fig. 21a. Each point represents the mean of eight membranes.

b) The effect of extended egg incubation (37.5°C/ r.h. 60%) on the growth of coliforms (originating from the faecal inoculum, Fig. 22a). Details as for Fig. 22a.

c) The effect of extended egg incubation (37.5°C/r.h. 60%) on the growth of enterococci (originating from the faecal inoculum, Fig 22a). Details as for Fig. 22a.

d) The effect of extended egg incubation (37.5°C/ r.h. 60%) on the growth of pseudomonads (originating from the faecal inoculum, Fig. 22a). Details as for Fig. 22a.

Fig. 22


a)
b)
12.0
10.0
8.0
6.0
4.0
2.0
< 2.0
Log10 no cfu/membranes
0 2 4 6 8 10 12 14 16 18 20
Time (days)

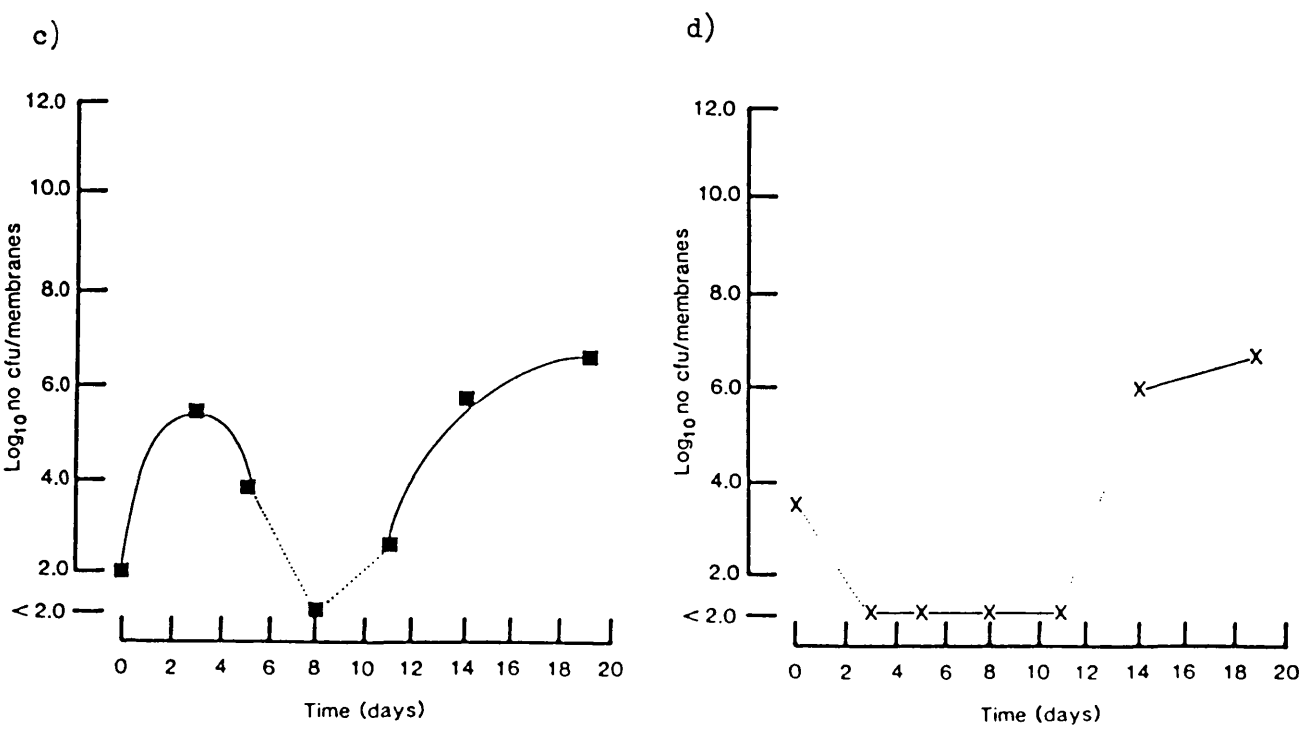

c)
d)
12.0
10.0
8.0
6.0
4.0
2.0
< 2.0
Log10 no cfu/membranes
0 2 4 6 8 10 12 14 16 18 20
Time (days)

Fig. 22 (cont)

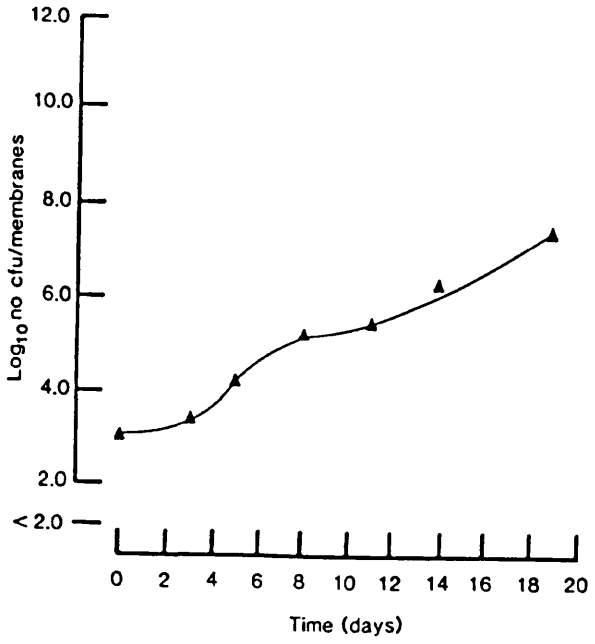
e) The effect of extended egg incubation (37.5°C/r.h. 60%) on the growth of Salmonella anatum (added to the faecal inoculum, Fig. 22a). Details as for Fig. 22a.

f) The effect of extended egg incubation (37.5°C/r.h. 60%) on the growth of micrococci (originating from the faecal inoculum, Fig. 22a. Details as for Fig. 22a.

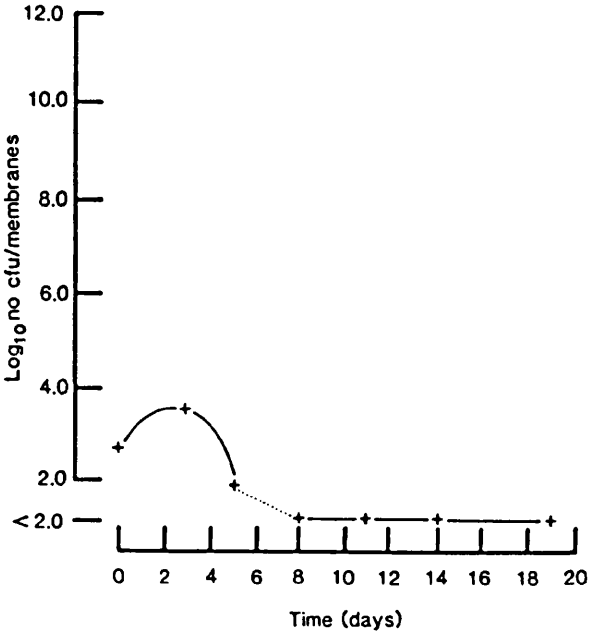
g) The effect of extended egg incubation (37.5°C/r.h. 60%) on the extent of contamination (total count) of the albumen. Details as for Fig. 22a.

Fig. 22 (cont)

e)



f)



g)

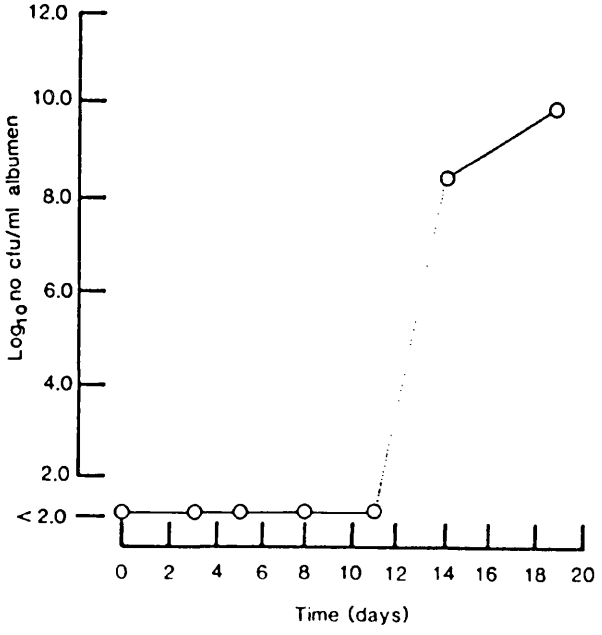


Fig. 23

a) The effect (on the membrane total count) of "storing" eggs (16°C/r.h. 75%) with a faecal-contaminated inner shell membrane prior to incubation (37°C/r.h. 60%). Stippled time span denotes period of "storage". Each point represents the mean of eight membranes.

b) The effect of "storage" on the growth of coliforms (originating from the faecal inoculum, Fig. 23a). Details as for Fig. 23a).

c) The effect of "storage" on the growth of enterococci (originating from the faecal inoculum, Fig. 23a). Details as for Fig. 23a.

d) The effect of "storage" on the growth of pseudomonads (originating from the faecal inoculum, Fig. 23a). Details as for Fig 23a.

Fig. 23

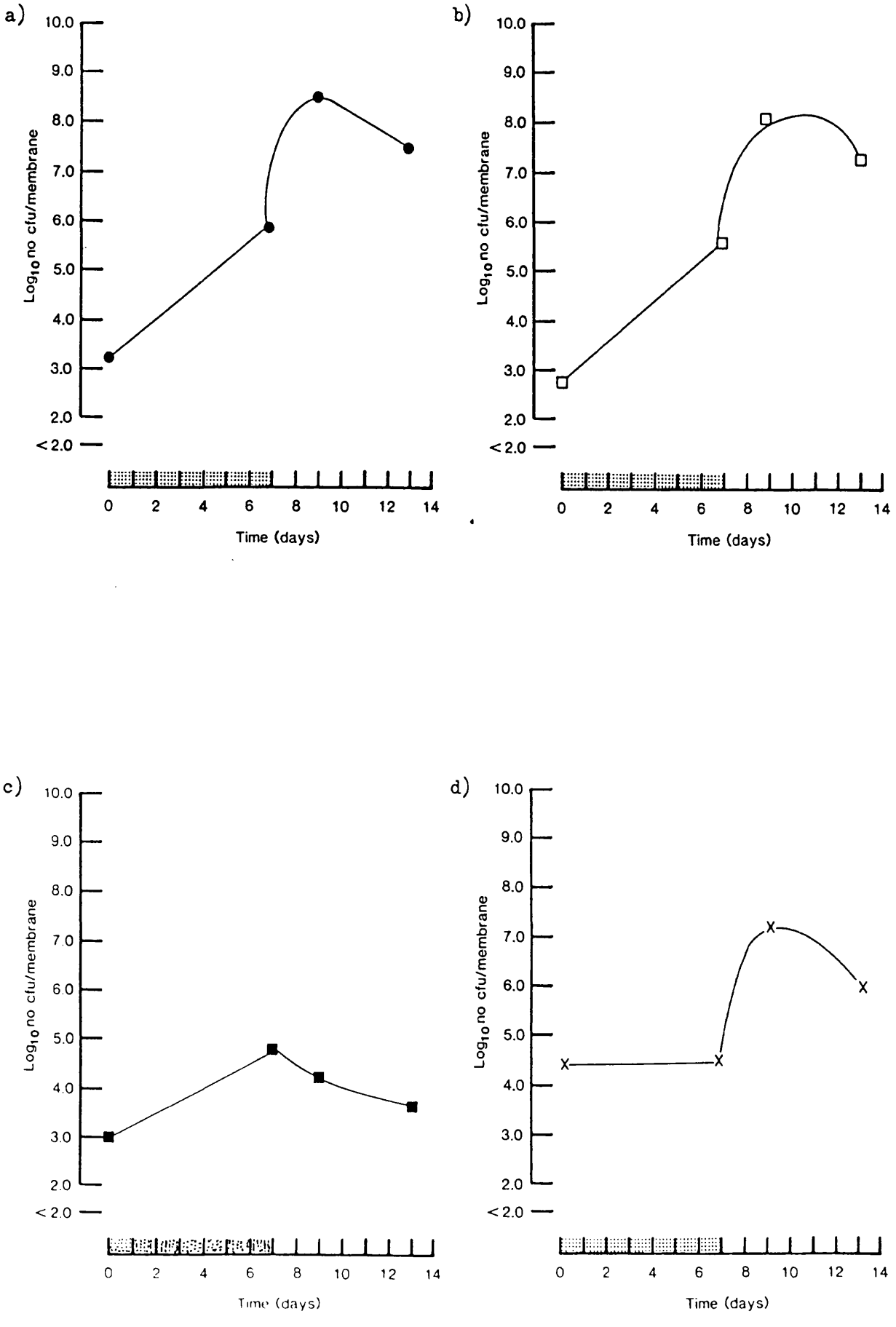

a)
b)
c)
d)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
< 2.0
Log10 no cfu/membrane
0 2 4 6 8 10 12 14
Time (days)

Fig. 23 (cont)

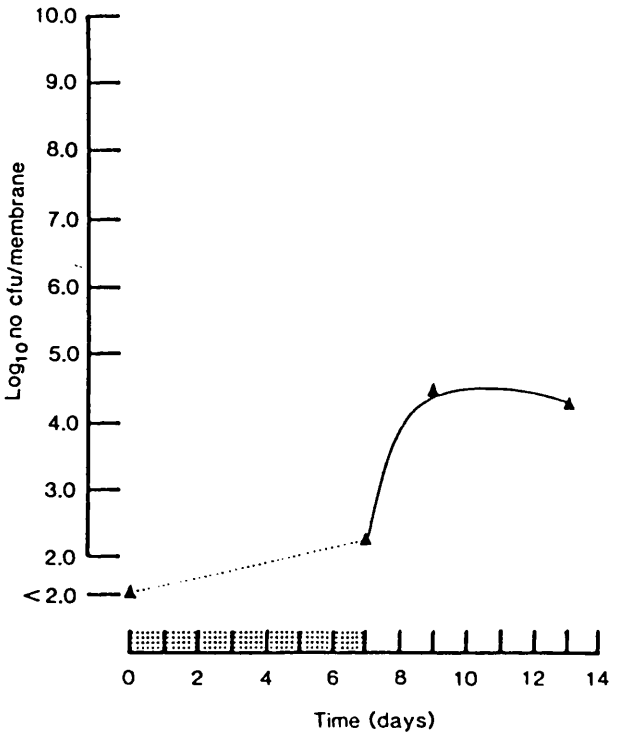
e) The effect of "storage" on the growth of Salmonella hadar (added to the faecal inoculum, Fig. 23a). Details as for Fig. 23a.

f) The effect of "storage" on the growth of micrococci (originating from the faecal inoculum, Fig. 23a). Details as for Fig. 23a.

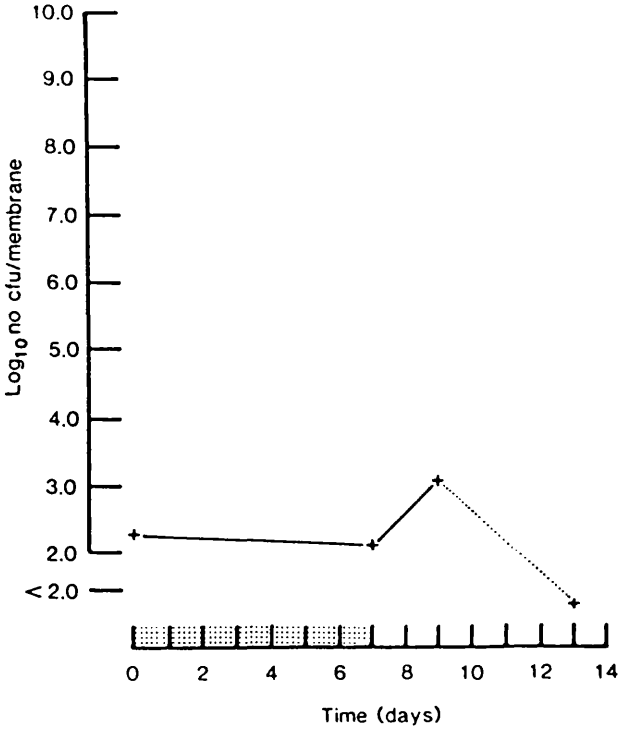
g) The effect of "storage" on the extent of contamination (total count) of the albumen. Details as for Fig. 23a.

Fig. 23 (cont)

e)



f)



g)

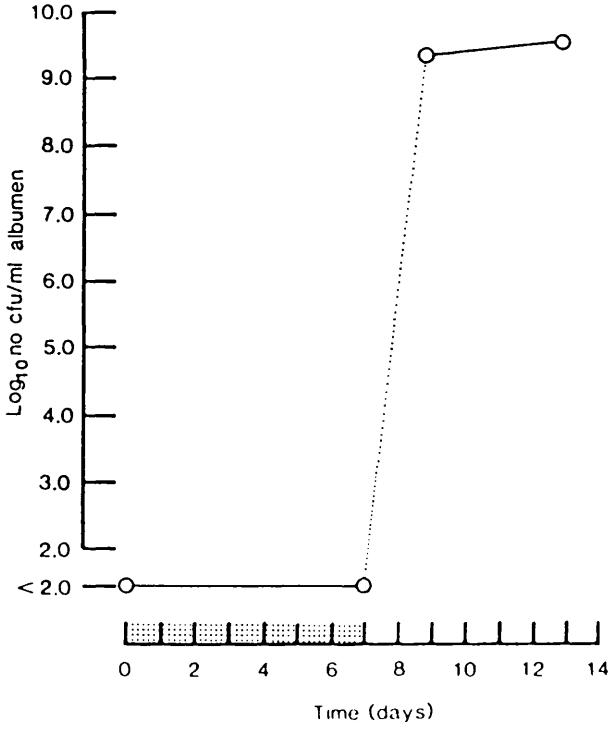


Fig. 24

a) The effect of inoculum level on the growth of Salmonella typhimurium when incubated (in vitro, 37.5°C) in hens' egg albumen (pH 8.62).

b) The effect of inoculum level on the growth of Salmonella goelitz when incubated (in vitro, 37.5°C) in hens' egg albumen (pH 8.62).

c) The effect of inoculum level and pH (cf Fig. 24a) on the growth of Salmonella typhimurium when incubated (in vitro, 37.5°C) in hens' egg albumen (pH 9.42).

d) The effect of inoculum level and pH on the growth of Salmonella goelitz when incubated (in vitro, 37.5°C) in hens' egg albumen (pH 9.42).

Fig. 24

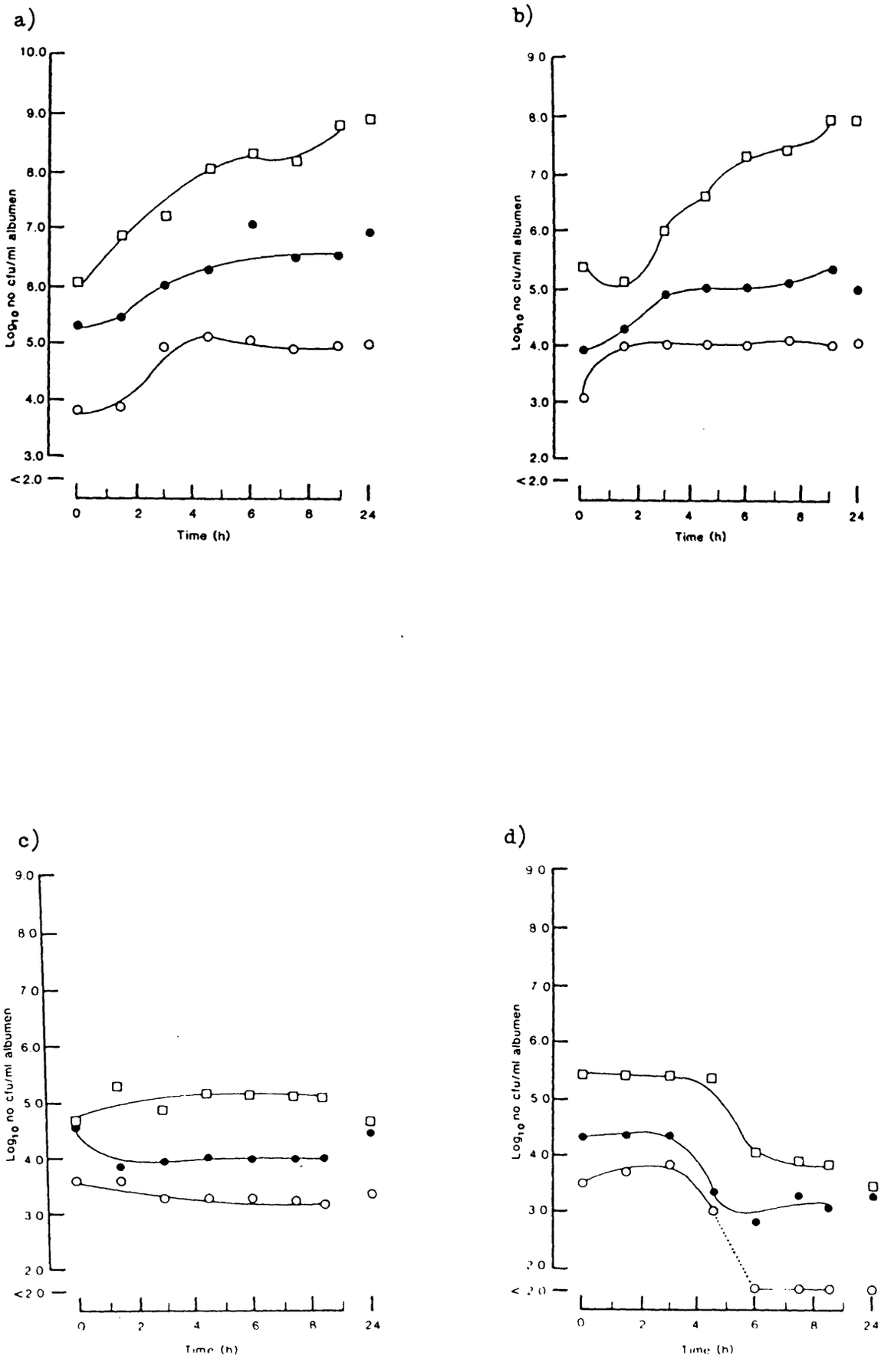

a)
b)
c)
d)
Log10 no cfu/ml albumen
Time (h)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
<2.0
0
2
4
6
8
24

Fig. 24 (cont)

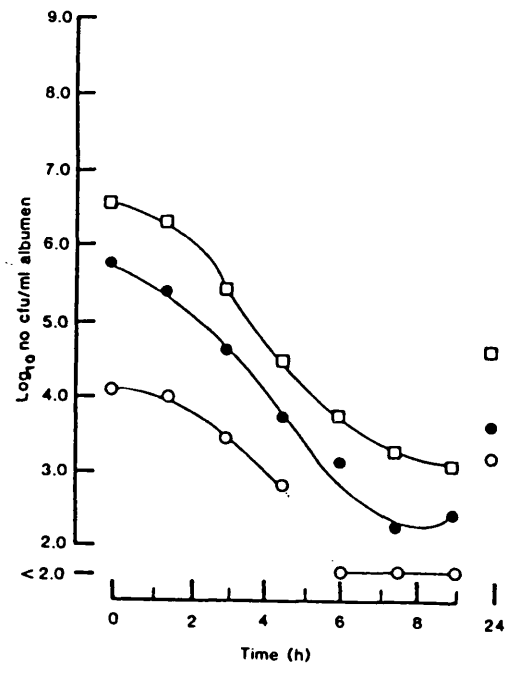
e) The effect of inoculum level on the growth of Salmonella anatum when incubated (in vitro, 37.5°C) in hens' egg albumen (pH 9.40).

f) The effect of inoculum level on the growth of Salmonella virchow when incubated (in vitro, 37.5°C) in hens' egg albumen (pH 9.40).

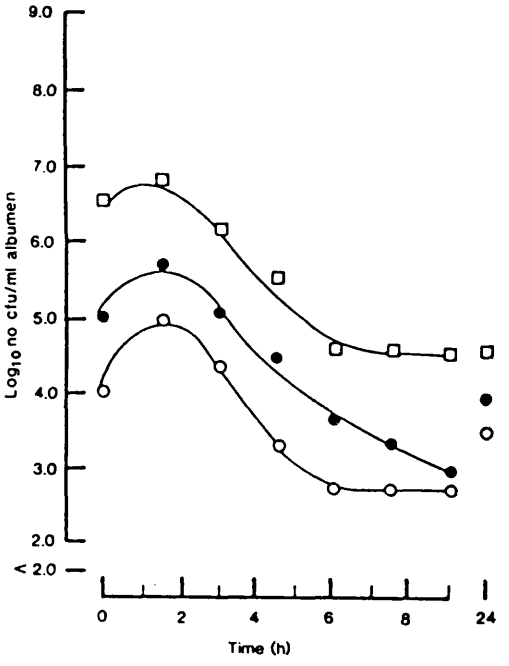
g) The effect of inoculum level on the growth of Salmonella hadar when incubated (in vitro, 37.5°C) in hens' egg albumen (pH 9.40).

Fig. 24 (cont)

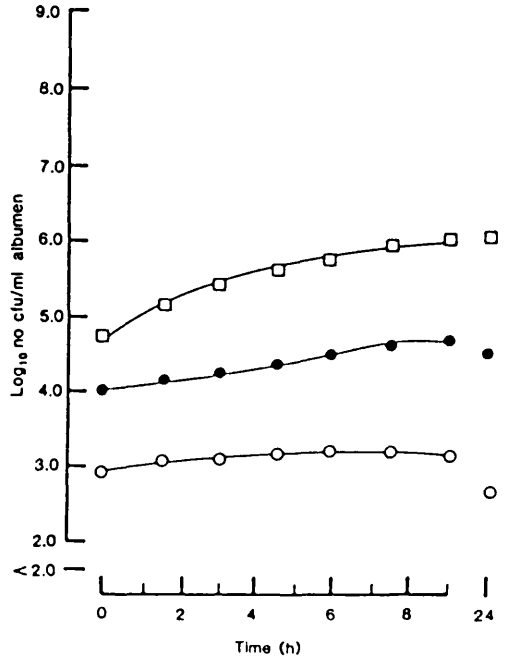
e)



f)



g)



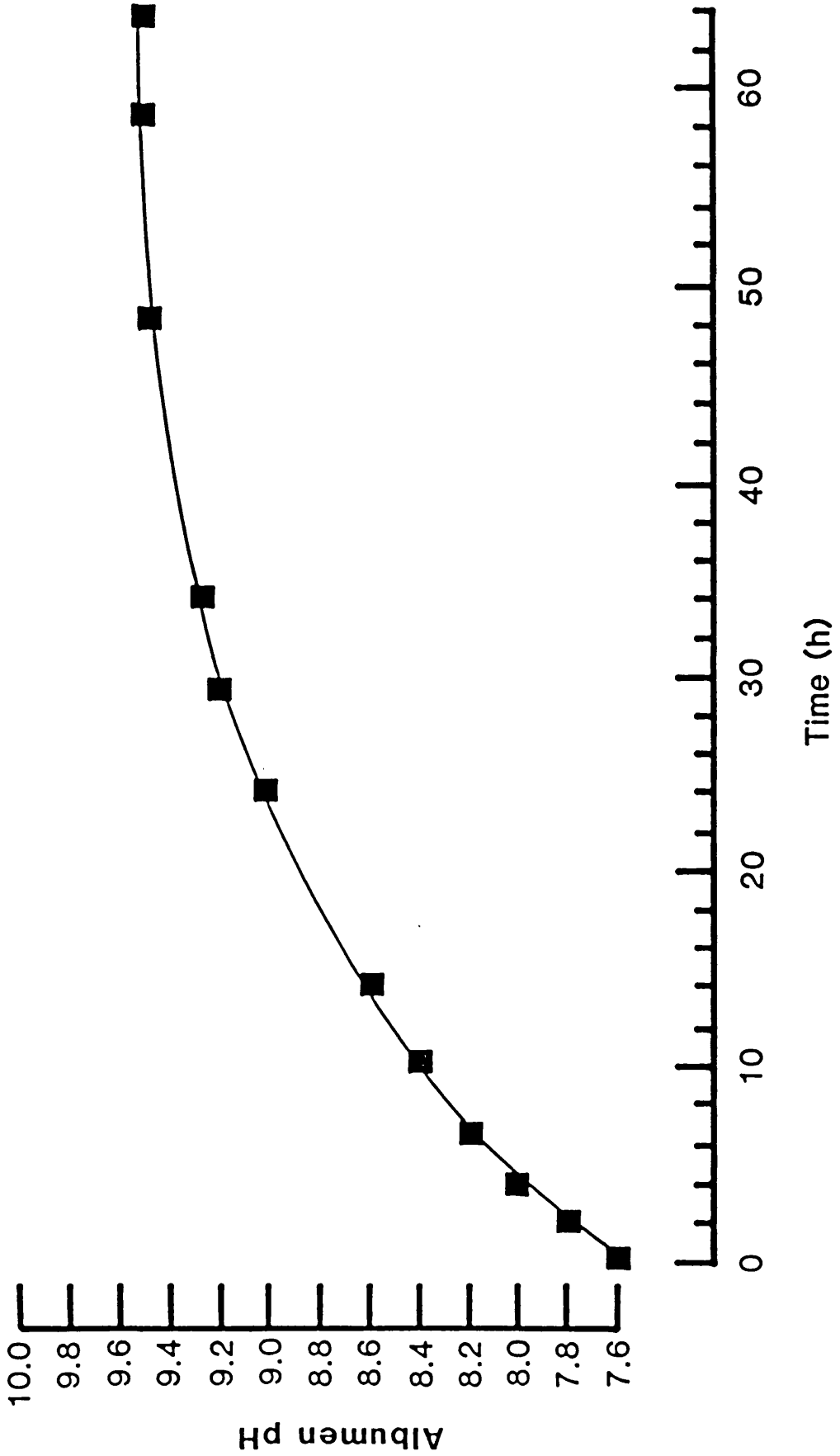


Fig. 25 The effect of incubation (37.5°C) on the pH of sterile albumen.

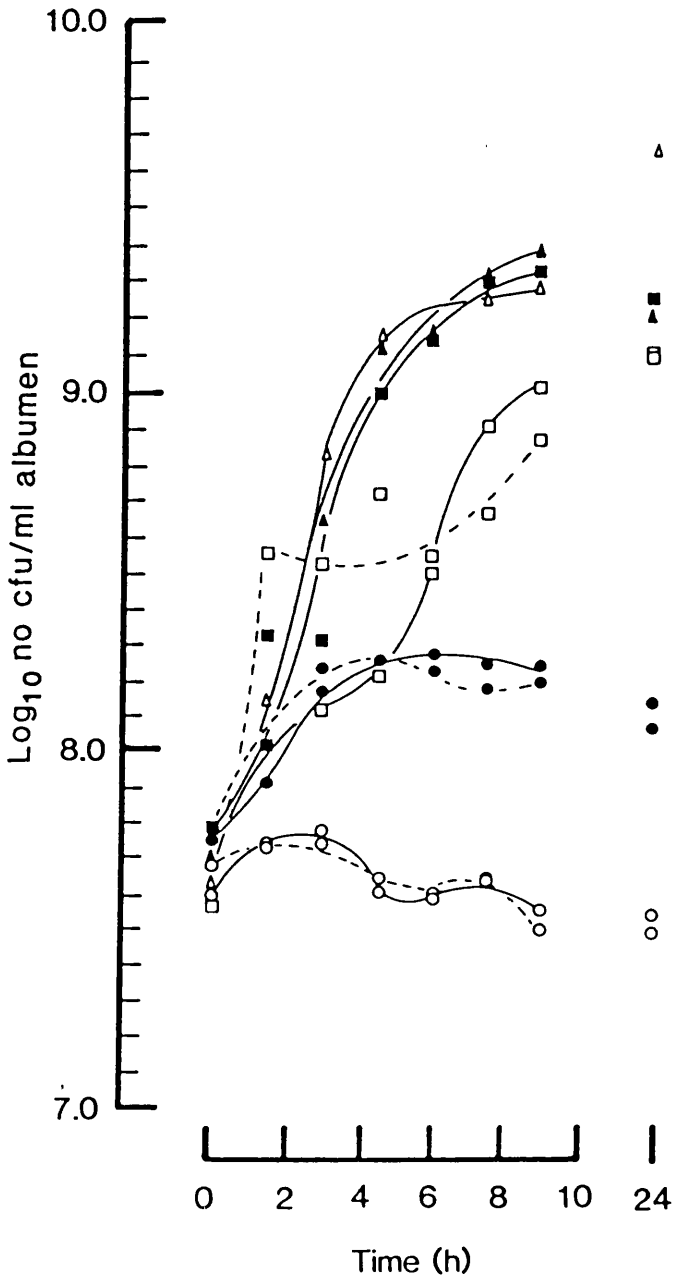


Fig. 26 The change in the antimicrobial efficacy of hens' egg albumen (taken from eggs incubated at 37.5°C/r.h. 60%) as shown by the growth of Salmonella hadar. Solid line, albumen from infertile eggs; broken line, albumen from fertile eggs. Day 0 of incubation, ○ ; Day 3, ●; Day 6, □; Day 10, ■; Day 18, Δ ; Day 21, ▲ . Each point represents the mean of 3 samples.

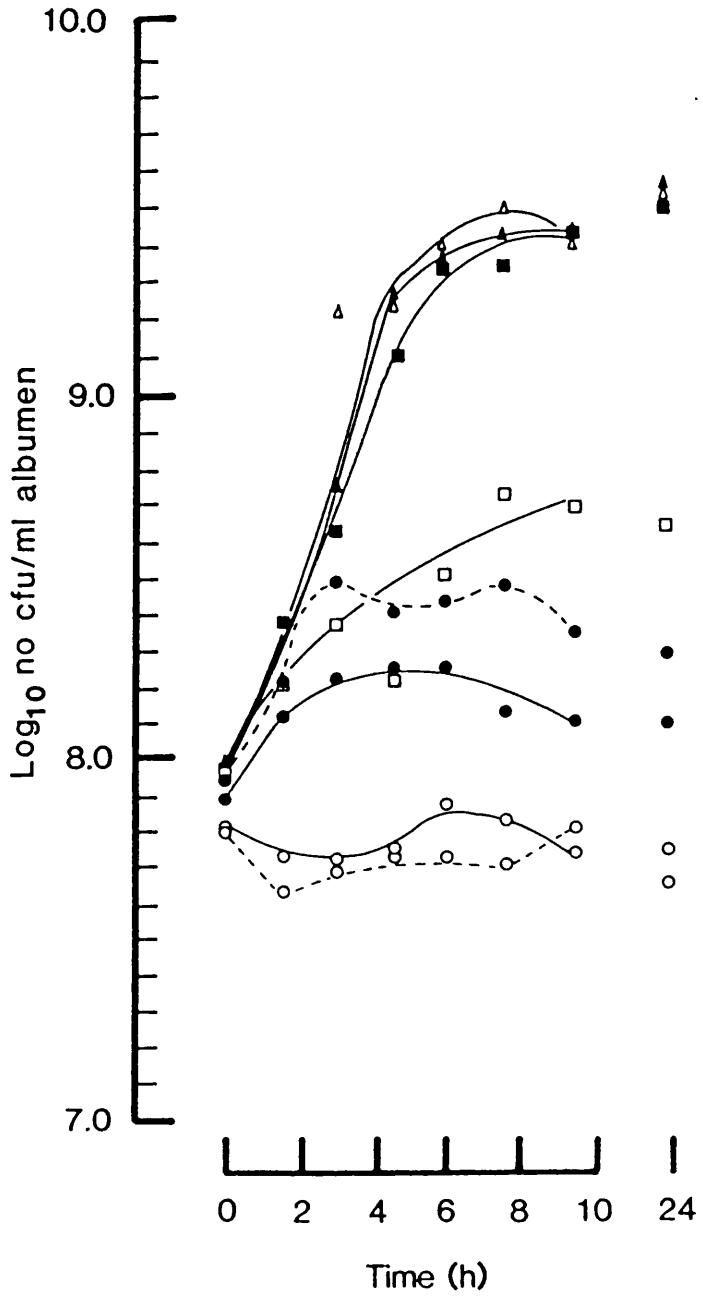


Fig. 27 The change in the antimicrobial efficacy of hens' egg albumen (taken from eggs incubated at 37.5°C/r.h. 60%) as shown by the growth of Salmonella typhimurium. Solid line, albumen from infertile eggs; broken line, albumen from fertile eggs. Day 0 of incubation, ○; Day 3, ●; Day 6, □; Day 10, ■; Day 18, △; Day 21, ▲. Each point represents the mean of 3 samples.

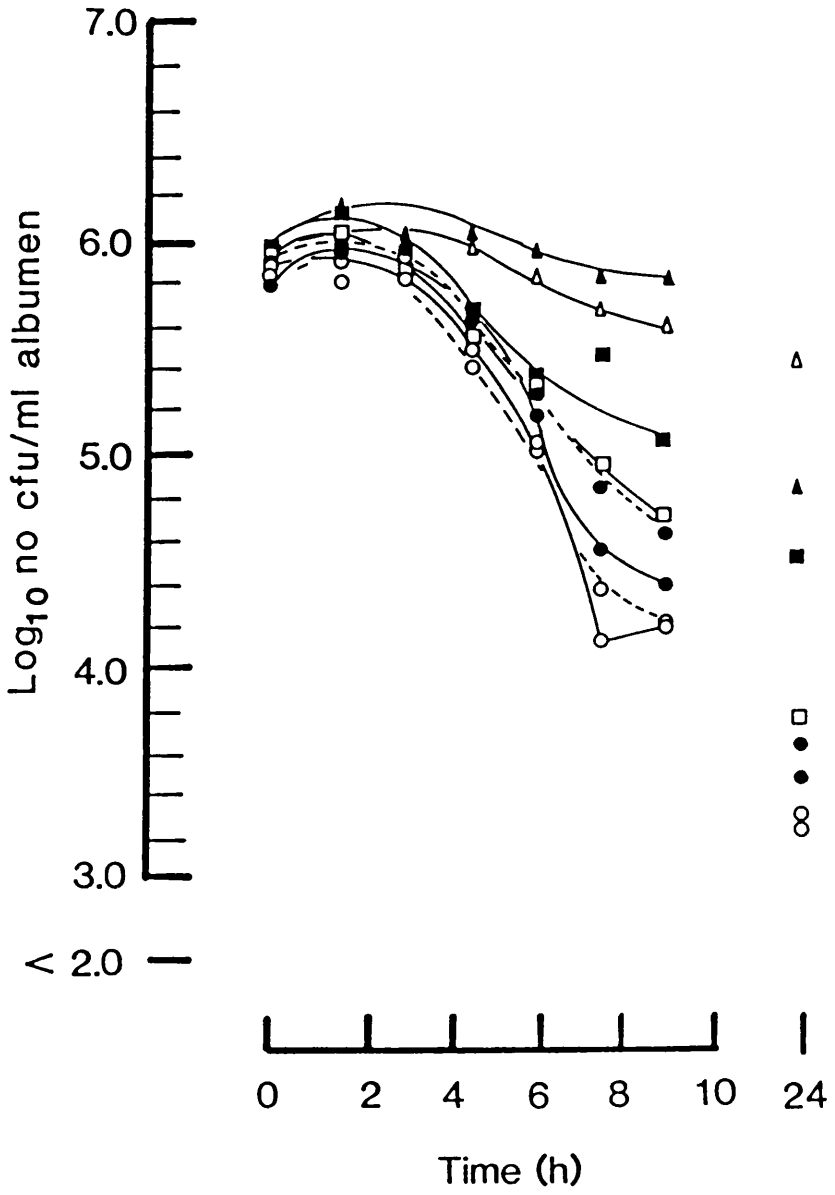


Fig. 28 The change in the antimicrobial efficacy of hens' egg albumen (taken from eggs incubated at 37.5°C/r.h. 60%) as shown by the growth of Salmonella hadar. Solid line, albumen from infertile eggs; broken line, albumen from fertile eggs. Day 0 of incubation, O; Day 3, ●; Day 6, □; Day 10, ■; Day 18, Δ; Day 21, ▲. Each point represents the mean of 3 samples.

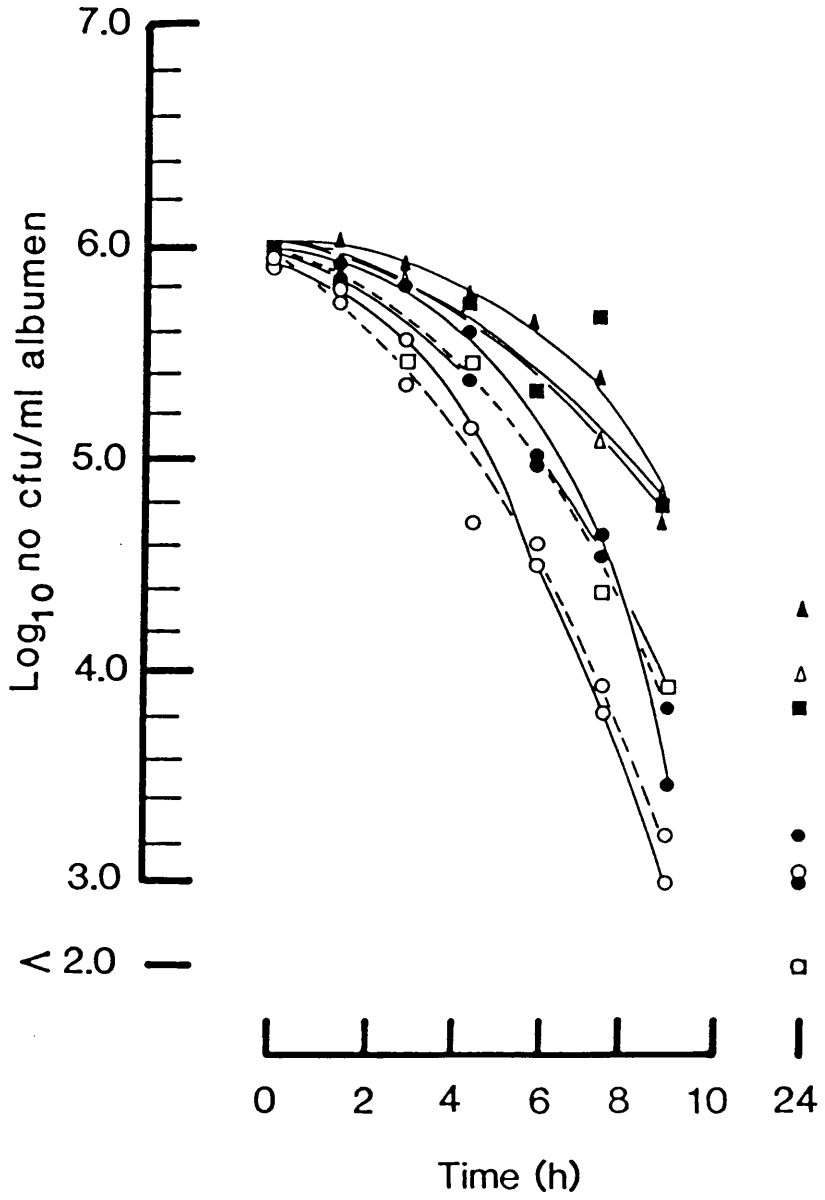


Fig. 29 The change in the antimicrobial efficacy of hens' egg albumen (taken from eggs incubated at 37.5°C/r.h. 60%) as shown by the growth of Salmonella typhimurium. Solid line, albumen from infertile eggs; broken line, albumen from fertile eggs. Day 0 of incubation, O; Day 3, ●; Day 6, □; Day 10, ■; Day 18, Δ; Day 21, ▲. Each point represents the mean of 3 samples.

Fig. 30

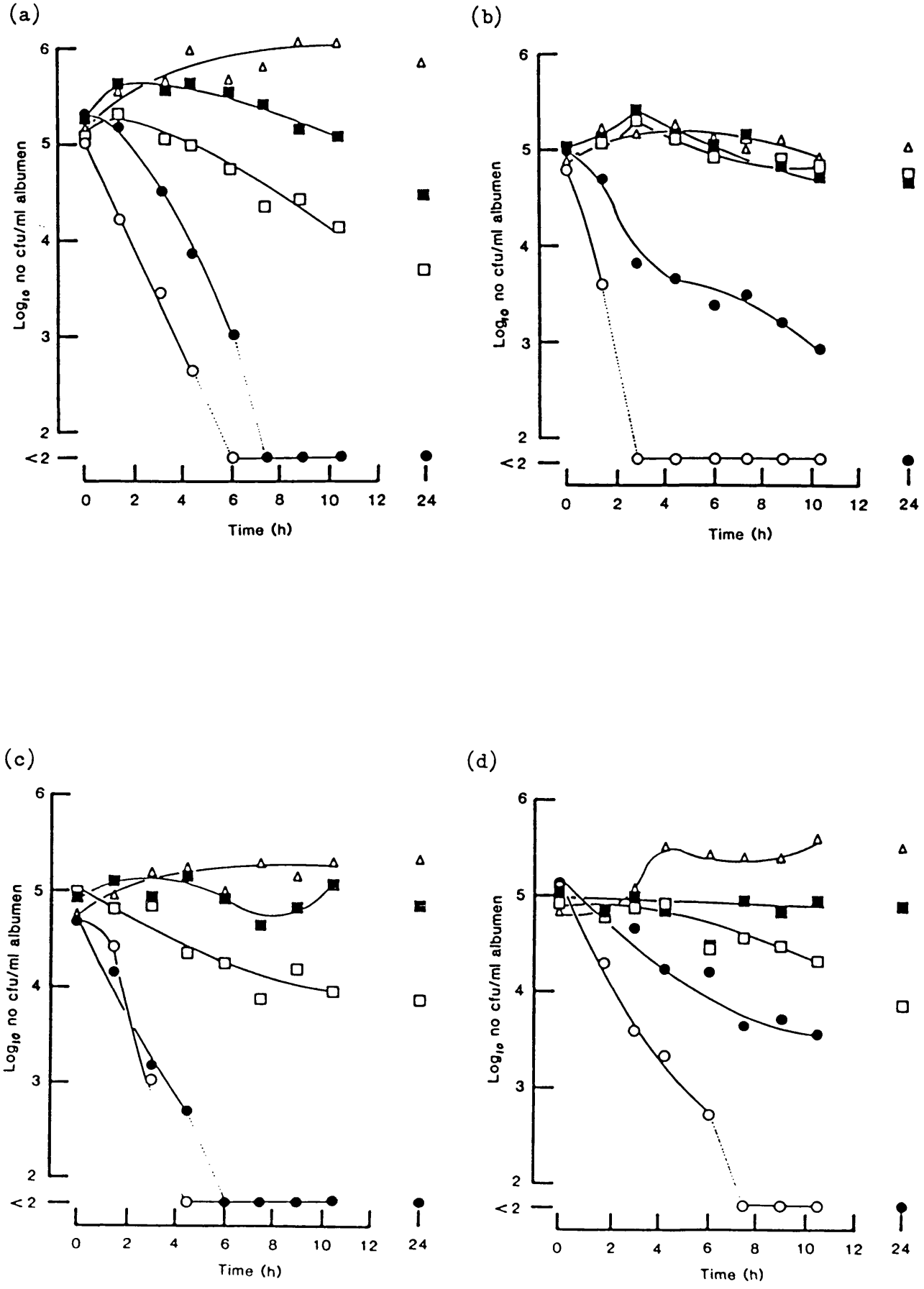
(a) Effect of incubation temperature on the growth of Salmonella anatum in hens' egg albumen. ○ - 44°C, ● - 39.5°C, □ - 30°C, ■ - 25°C, Δ - 6.5°C.

(b) Effect of incubation temperature on the growth of Salmonella kedogan in hens' egg albumen. Legend as for Fig. 30a.

(c) Effect of incubation temperature on the growth of Salmonella virchow in hens' egg albumen. Legend as for Fig. 30a.

(d) Effect of incubation temperature on the growth of Salmonella hadar in hens' egg albumen. Legend as for Fig. 30a.

Fig. 30



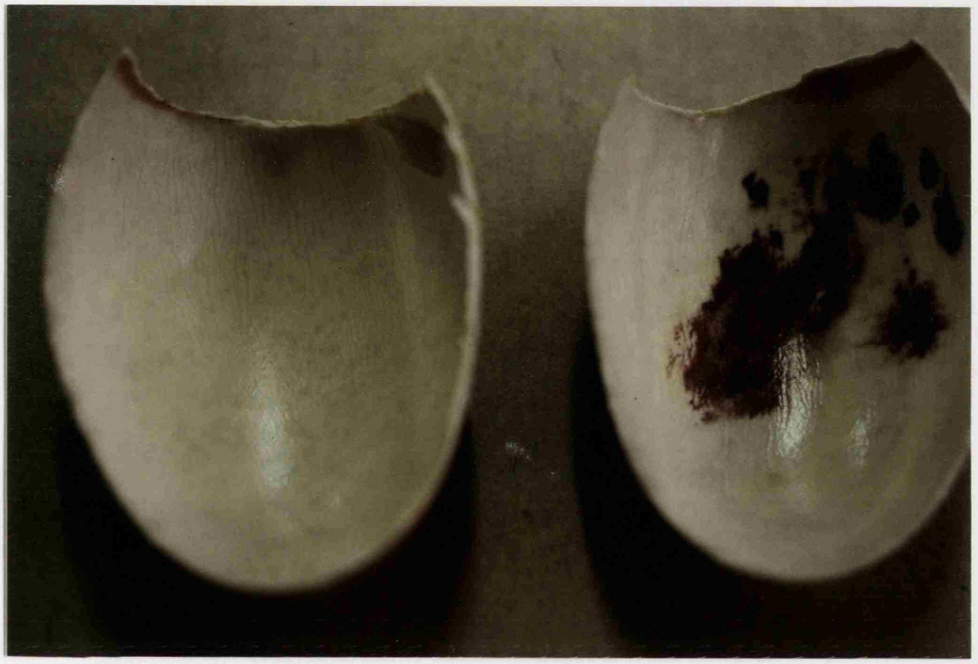


Plate 19. Spots of formazan - formed by the microbial reduction of 2, 3, 5-triphenyltetrazolium chloride - were a feature of the membranes underlying immature cuticles (right hand section) whereas the mature cuticle (left hand section) of the same egg restricted bacterial penetration.

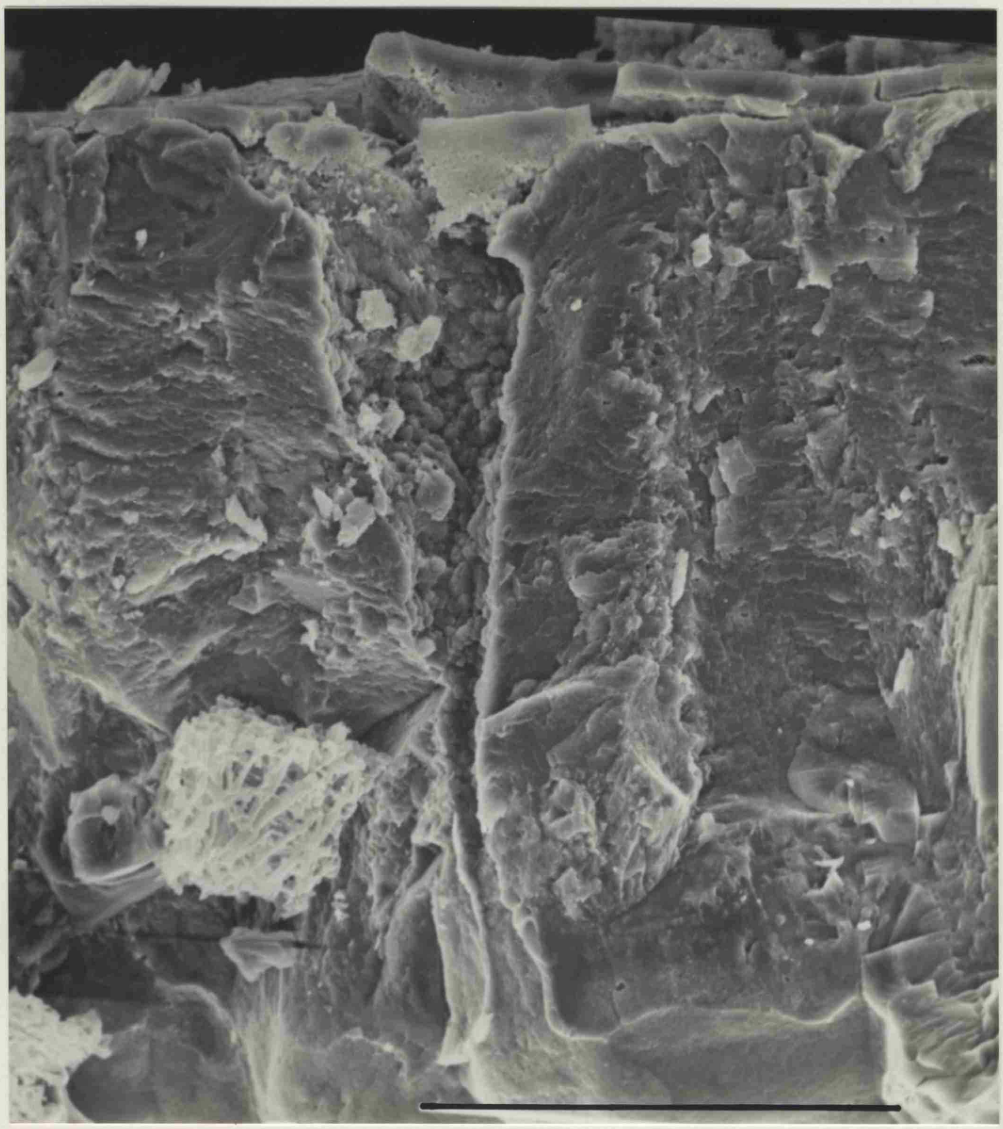


PLATE 20. Scanning electron micrograph of an oversized or "patent" pore.

Bar marker 100µm.

PLATE 21. Casts (formed by impregnating the shell with resin) of pore canals found within one square centimetre of hen eggshell. Note the diversity of pore diameters and configurations.
Bar marker 100μm.

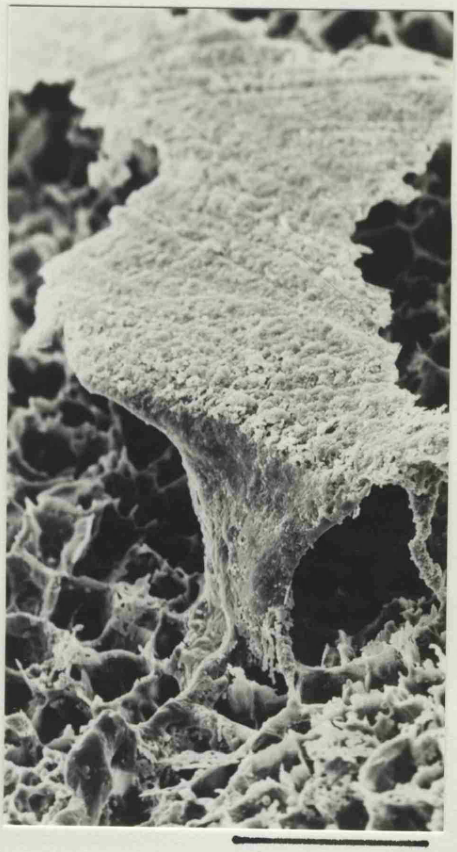


PLATE 22.

a) TEM section showing Fe^{3+} (F) adhering to the mantle of an inner shell membrane fibre. Bar marker 1μm.

b) Shell membranes stained with colloidal iron - taken up through pore canals by temperature differentail dipping.


F

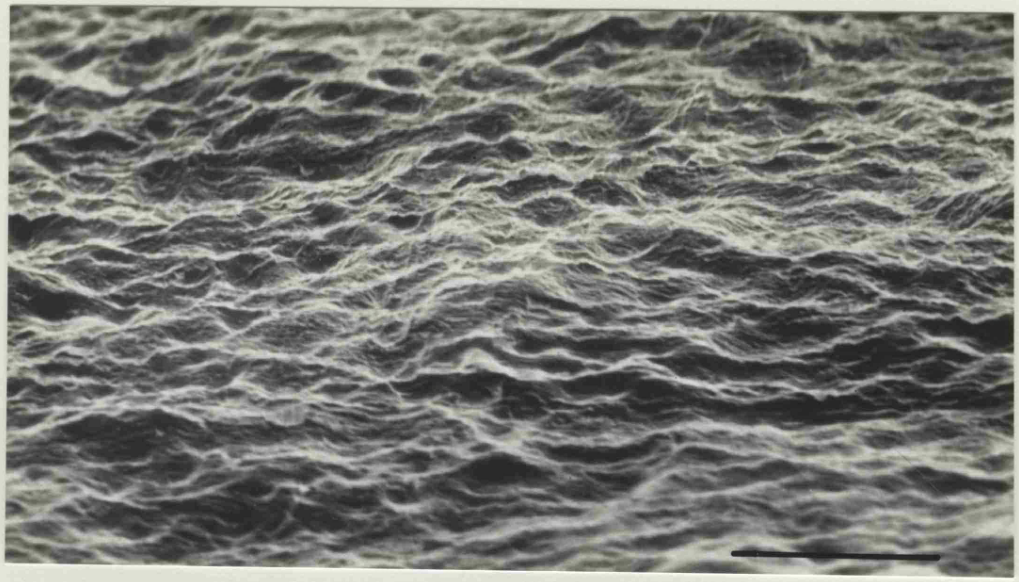

27-28

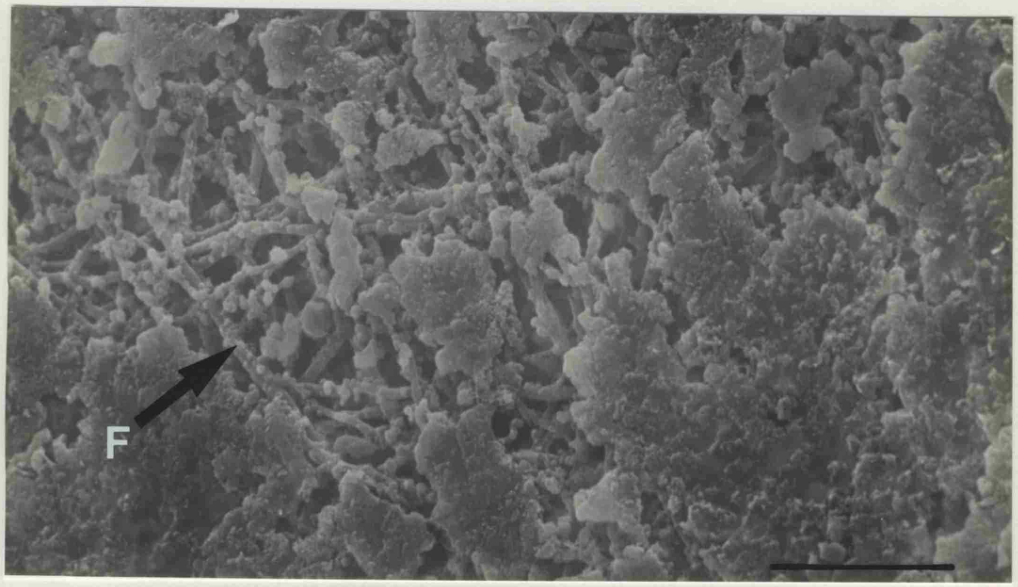
PLATE 23.

a) Scanning electron micrograph of the limiting membrane of an incubated (Day 0) fertile hen's egg.
Bar marker 10μm.

b) Scanning electron micrograph of the limiting membrane (fertile hen's egg) after six days incubation. The underlying fibres (F) are clearly visible. This was associated with an increased permeability of the shell integument to 0_2 (see Fig. 18).
Bar marker 10μm.

c) Scanning electron micrograph of the limiting membrane (fertile hen's egg) after 18 days incubation. The limiting membrane has almost completely deteriorated.
Bar marker 10μm.




F

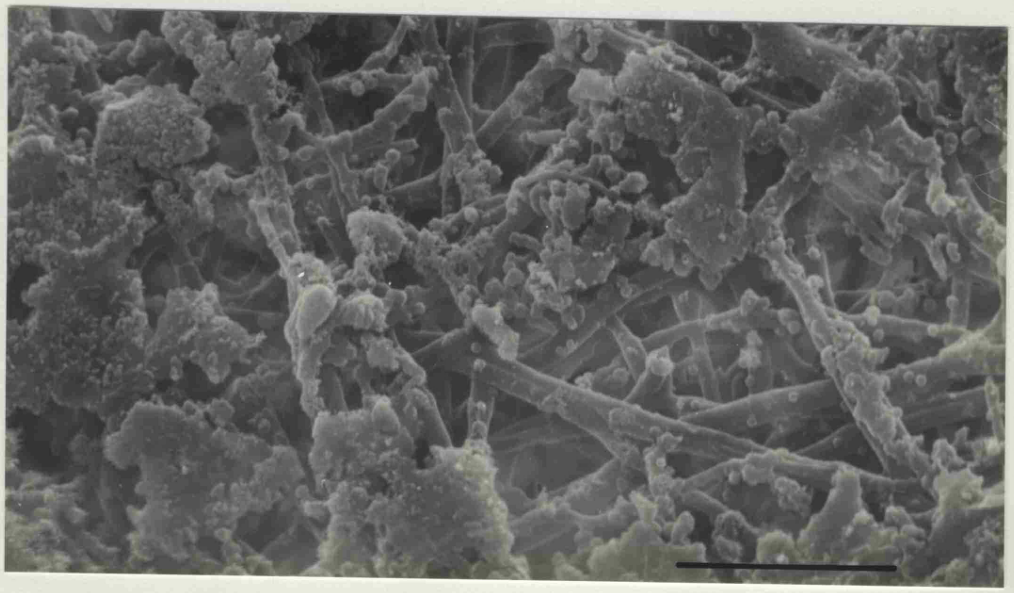


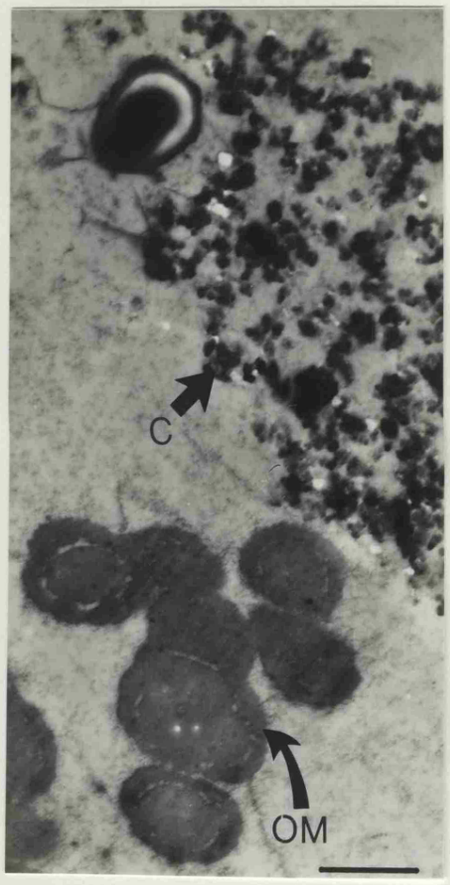
PLATE 24. Bacterial invasion of the inner shell membrane of incubated (37.5°C/r.h. 60%) hen's eggs

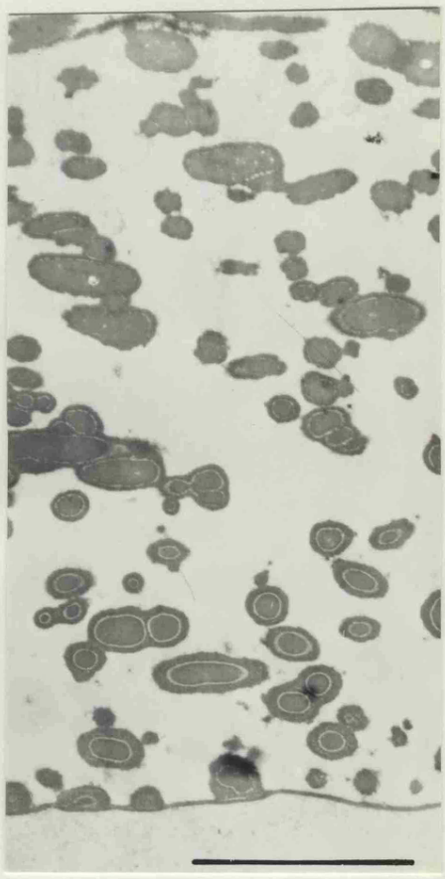
a) TEM of faecal material (mixed with carbon black - C) on the outer membrane fibres (OM) of the inner shell membrane. Corresponds to Fig. 21a - Time 0 of incubation.
Bar marker 1µm.

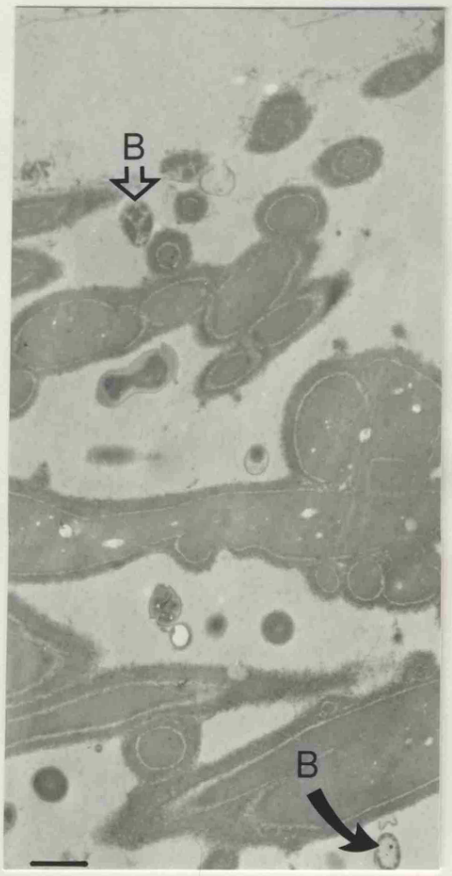
b) TEM section of the inner shell membrane - there is no evidence of bacterial penetration. Corresponds to Fig. 21a - Time 0 of incubation.
Bar marker 10µm

c) The outer section of the inner shell membrane, showing bacterial (B) penetration following two days incubation (Fig. 21a).
Bar marker 1µm

d) The inner section of the inner shell membrane showing bacteria (B) in the vicinity of the limiting membrane (two days incubation, Fig. 21a).
Bar marker 1µm.


C
OM




B
B

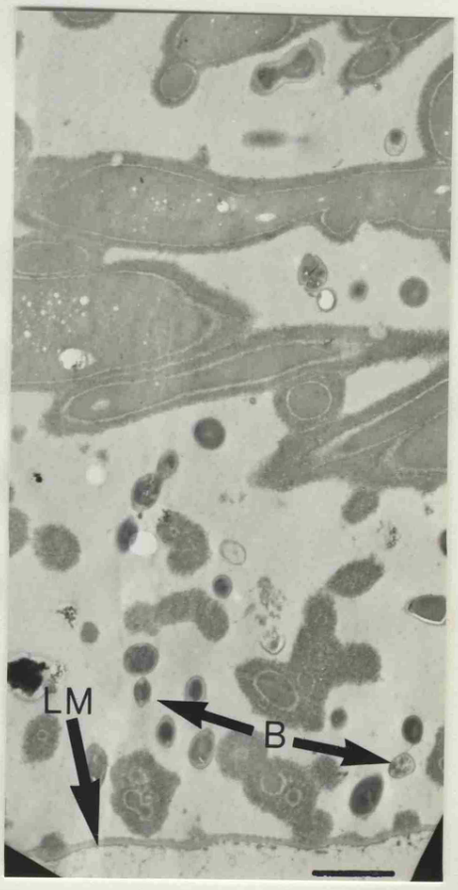
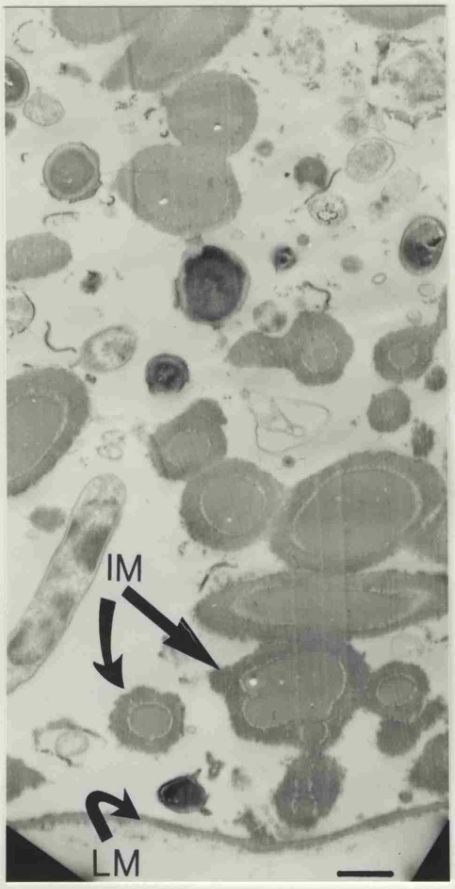

LM
B

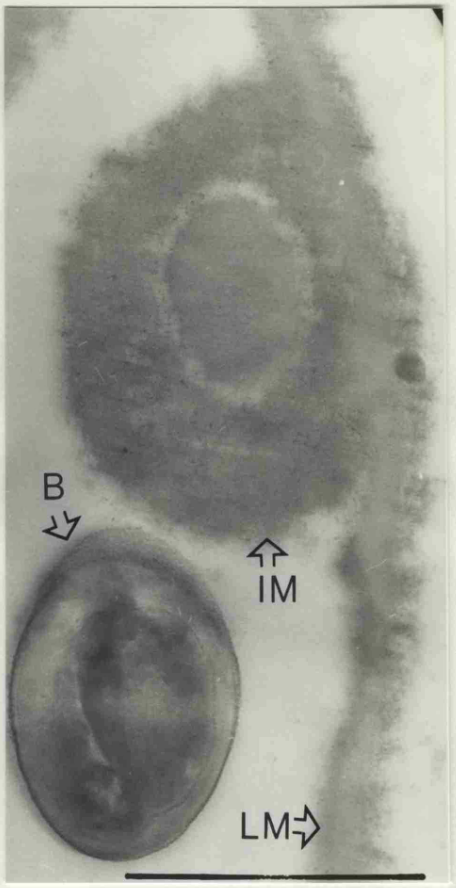
PLATE 25 Bacterial invasion of the inner shell membrane of incubated (37.5°C/r.h. 60%) hens' eggs

a) The inner section of the inner shell membrane after six days incubation (Fig. 21a). There is no evidence of bacterial degradation of the membrane fibres (IM) or of accumulation against the limiting membrane (LM).
Bar marker 1μm.

b) Bacterium in close proximity to an inner membrane fibre (IM) and the limiting membrane (LM) - no evidence of interaction.
Bar marker 1μm.

c) After six days incubation (Fig. 21a) there was evidence of bacterial cell "debri" (B). Limiting membrane (LM), inner membrane fibre (IM).
Bar marker 1μm.


IM
LM


B
IM
LM

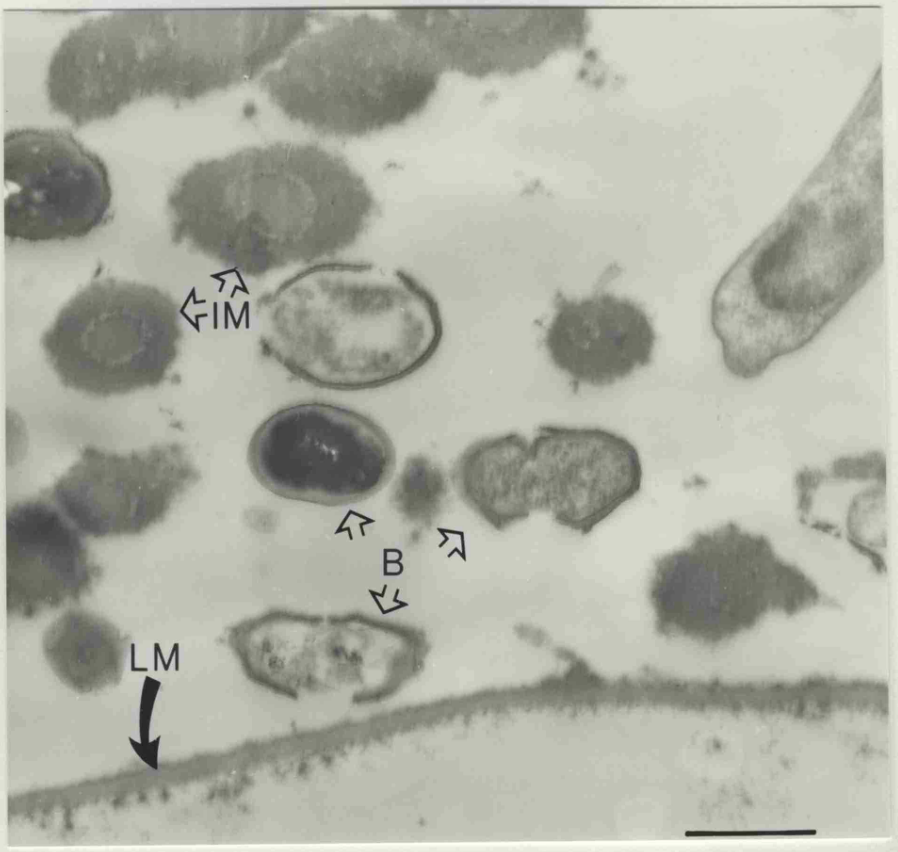
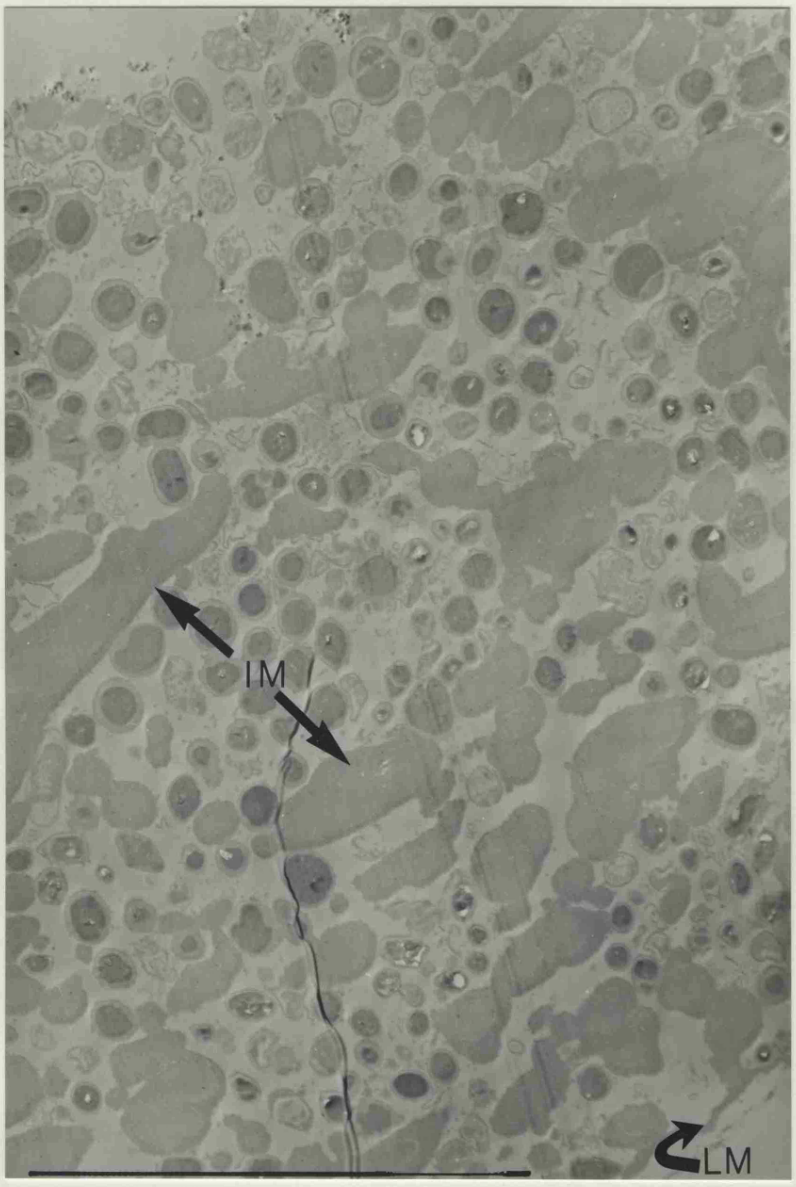

IM
B
LM

PLATE 26. Bacterial invasion of the inner shell membrane of incubated (37.5°C/r.h. 60%) hens' eggs

a) After 20 days incubation (Fig. 21a) the spaces between the inner membrane fibres (IM) were densely packed with microorganisms. Limiting membrane (LM). Bar marker 10µm.

b) Shows a possible interaction (I) between an inner membrane fibre (IM) and one of the many bacteria (B). Bar marker 1µm.


IM
LM

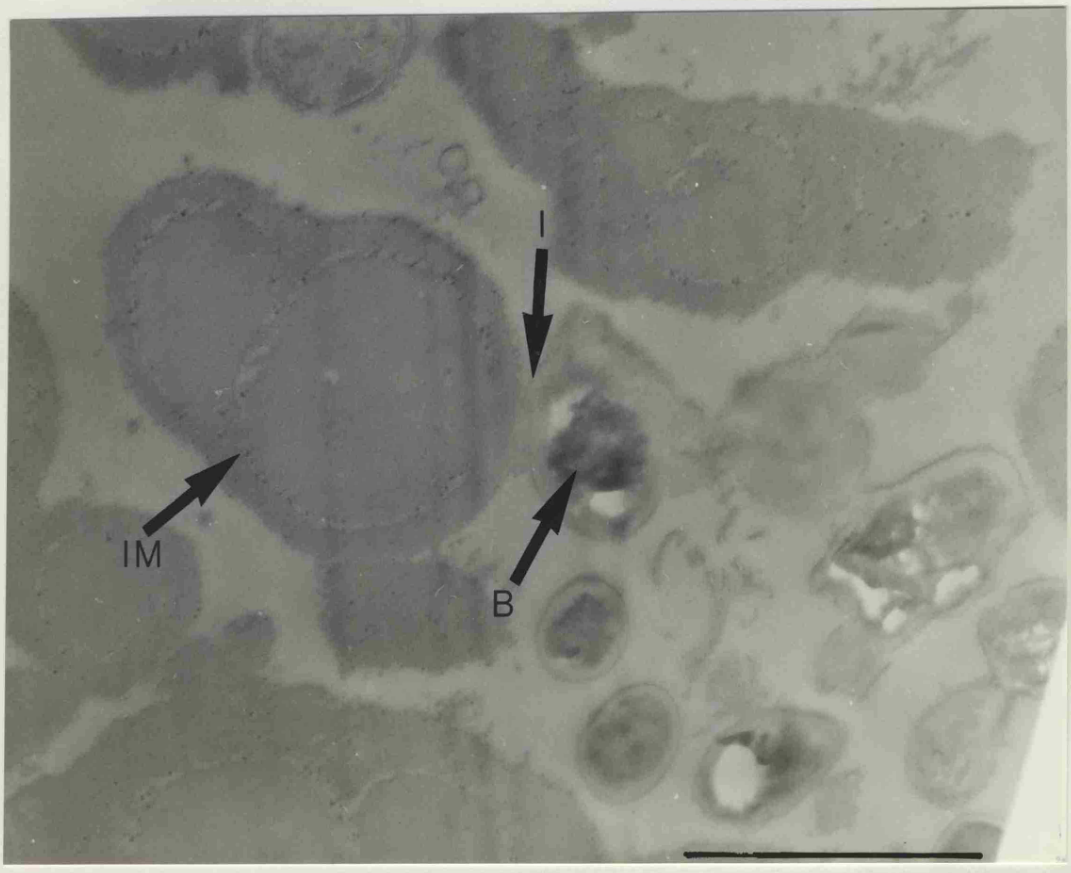

I
IM
B

PLATE 27. Evidence for microorganisms interacting with the membrane fibres or limiting membrane

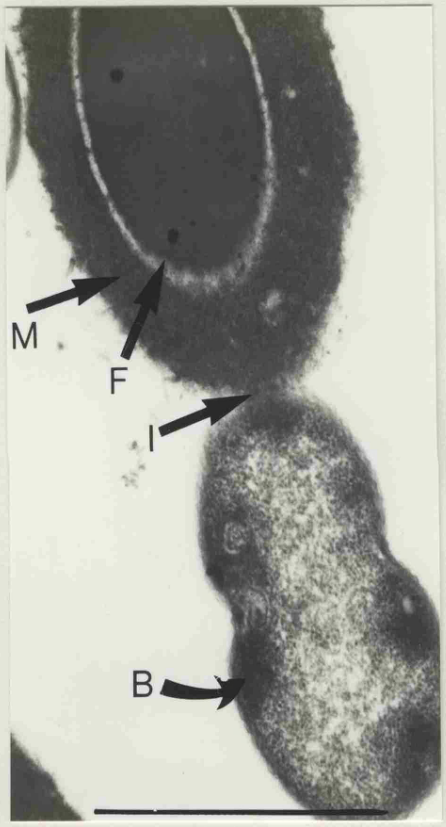
a) Zone of interaction (I) between a bacterium (B) and the mantle (M) of an inner membrane fibre (F).
Bar marker 1μm.

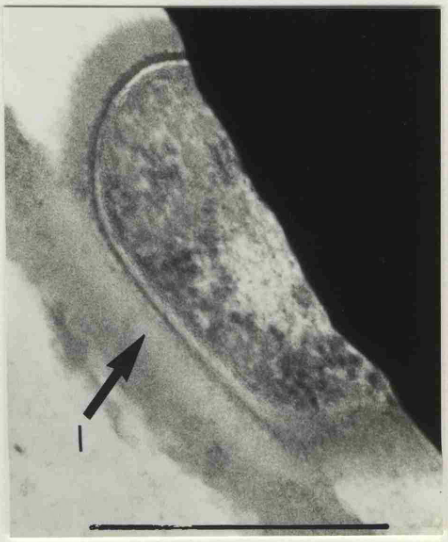
b) Zone of interaction (I) and apparent degradation between a bacterium and the limiting membrane.
Bar marker 1μm.

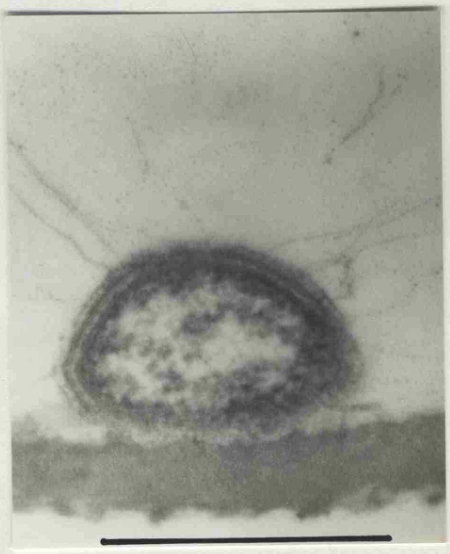
c) Microorganism resting against the limiting membrane.
Bar marker 1μm.

d) Microorganism resting against the limiting membrane.
Bar marker 1μm.

e) Microorganism resting against the limiting membrane.
Bar marker 1μm.


M
F
I
B


I



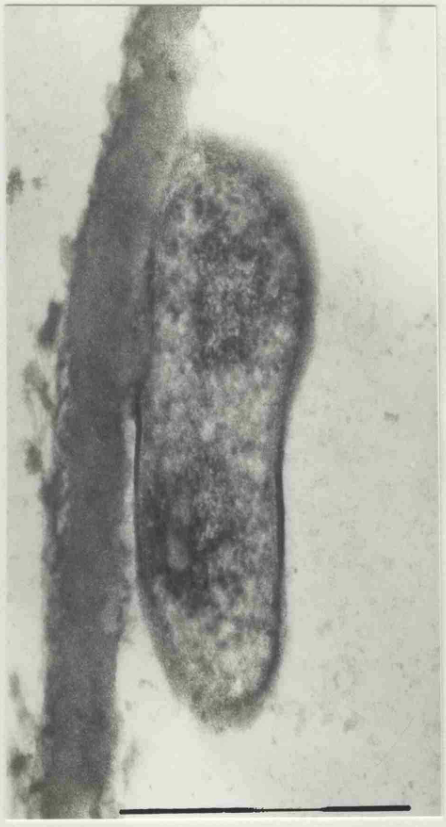


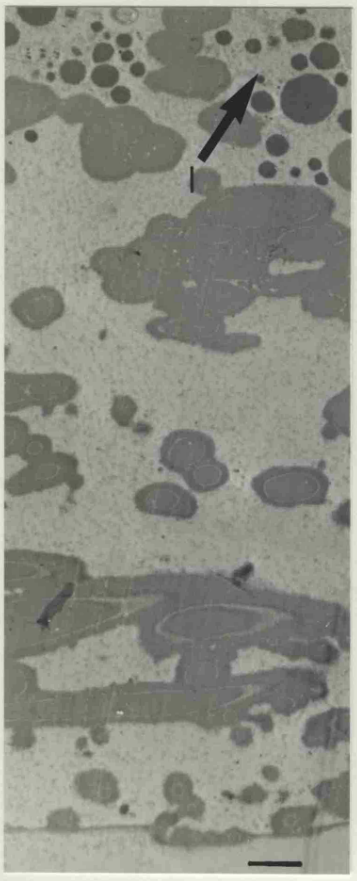
PLATE 28. Bacterial invasion of the inner shell membrane of "stored" (16°C/r.h. 75%) hens' eggs

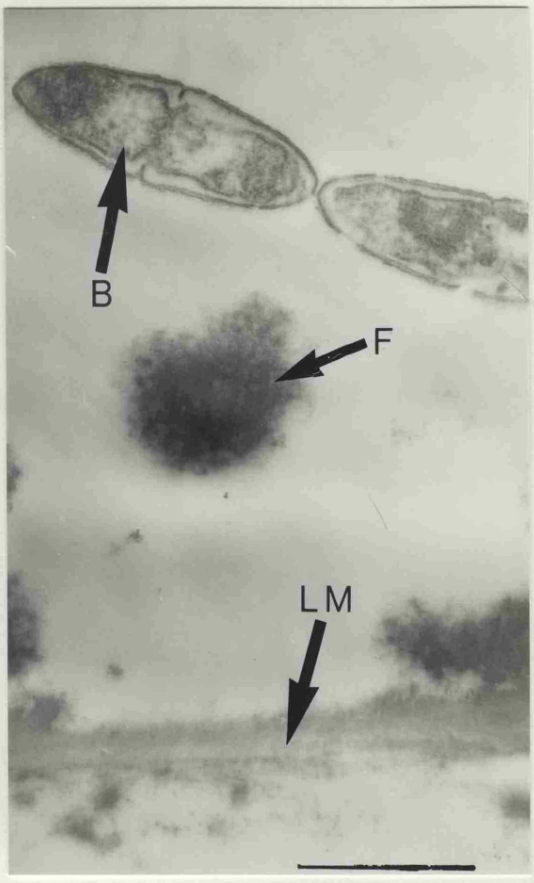
a) TEM section showing the inoculum (I) on the outer surface of the inner membrane immediately following inoculation - Fig. 23a. The speckled appearance of the membrane is due to poor uptake of stain.
Bar marker 1µm.

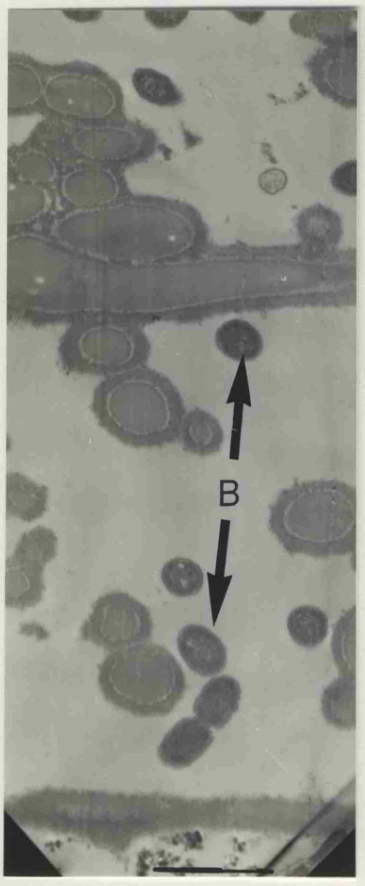
b) After one days incubation (Fig. 23a) bacteria (B) had reached the vicinity of the limiting membrane (LM). Inner membrane fibre (IM).
Bar marker 1µm

c) Bacteria (B) in the inner section of the inner membrane (one days incubation, Fig. 23a).
Bar marker 1µm.

d) Bacteria (B) in the outer section of the inner membrane (one days incubation, Fig. 23a).
Bar marker 1µm.


I


B
F
LM


B


B

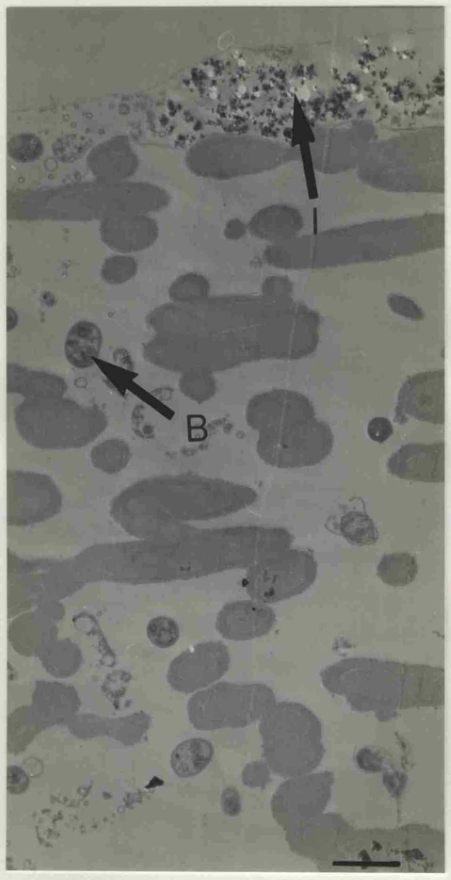
PLATE 29. Bacterial invasion of the inner shell membrane of "stored" ($16^{o}C$/r.h. 75%) hens' eggs

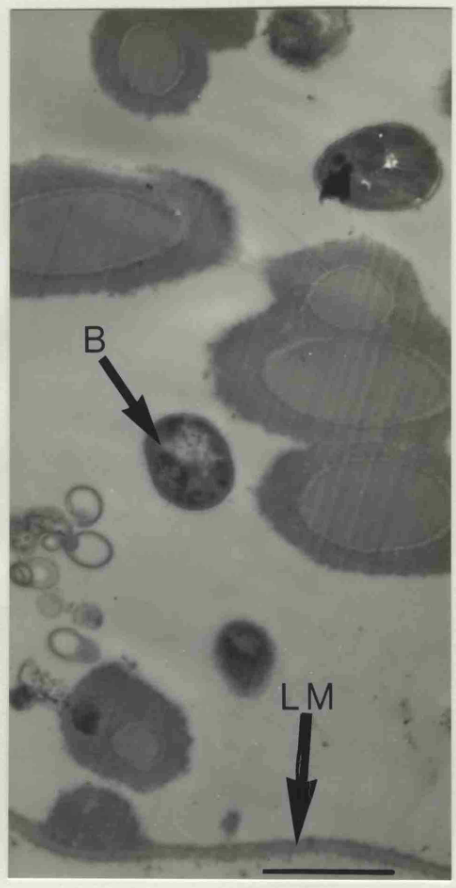
a) Outer section of the inner membrane after four days "storage" (Fig. 23a). Site of inoculation (I), bacteria (B).
Bar marker 1μm.

b) Inner section of the inner membrane after four days "storage" (Fig. 23a). Microorganisms (B), limiting membrane (LM).
Bar marker 1μm.

c) Microorganism resting against the limiting membrane (LM) - no evidence of interaction. Four days "storage" - Fig. 23a.
Bar marker 1μm.

d) Microorganisms in the vicinity of the limiting membrane (LM) - no evidence of interaction. Four days "storage" - Fig. 23a.
Bar marker 1μm.


I
B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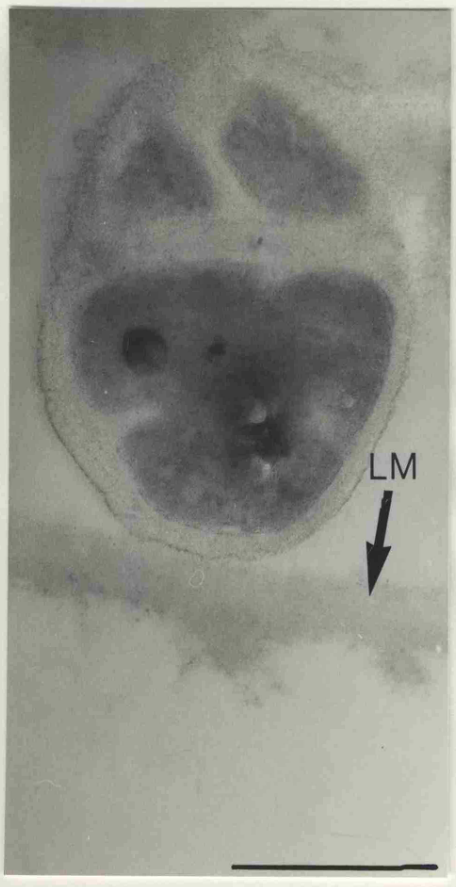
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PLATE 30. Bacterial invasion of the inner shell membrane of "stored" ($16^{\circ}C$/r.h. 75%) hens' eggs

a) Bacterial cell "debri" (B) in the vicinity of the limiting membrane (LM). Seven days "storage" - Fig. 23a. Bar marker 1μm.

b) Microorganisms resting against the limiting membrane (LM). No evidence of interaction. Seven days "storage" - Fig. 23a. Bar marker 1μm.

c) Section through the heavily contaminated inner shell membrane of an egg "stored" for seven days (Fig. 23a). Site of inoculum (I), limiting membrane (LM). Bar marker 10μm.


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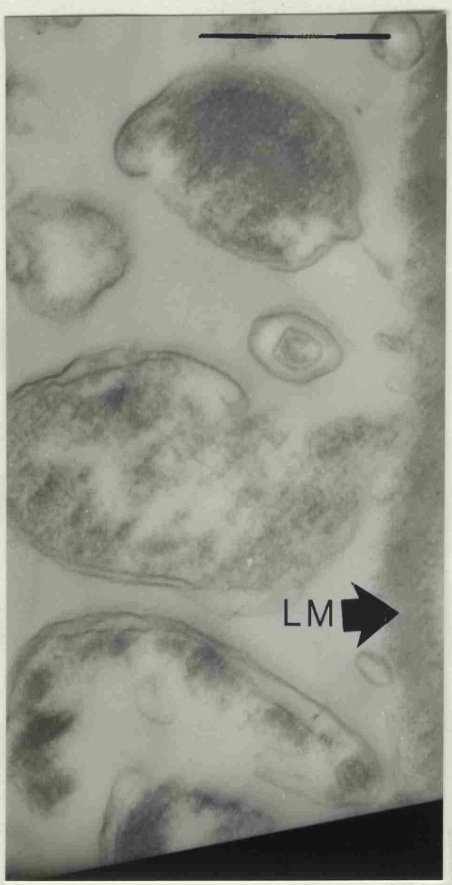
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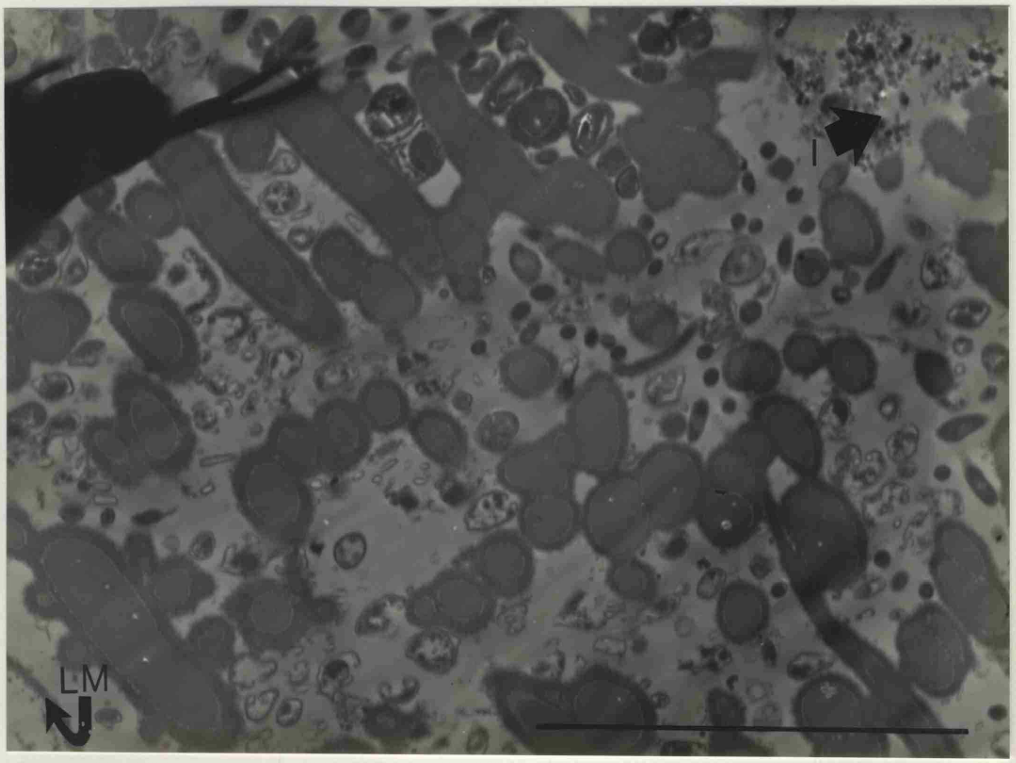
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DISCUSSION

DISCUSSION

The introduction of intensive farming (over 440 million poultry were produced in 1980 for the UK meat market - Anon, 1981) has brought economic benefits to the consumer. It has led also to a marked increase in infectious poultry diseases, in particular salmonellosis (Lee, 1973) the causative organisms of which are often passed on to the consumer. Although the same Salmonella serotype may infect both poultry and the consumer it is assessed by the relevant authorities in totally different ways. The poultry producers assess disease almost solely on an economic basis - loss of profit through death, for example - whereas public health assessment is subject to other criteria.

At one time the two poultry-specific serotypes, Salmonella gallinarum, and S. pullorum, regularly infected young flocks causing 25% mortality with not infrequent infection of the egg contents oviducally in laying flocks, a rare occurrence with other serotypes (Adler, 1965). As large scale poultry farms came into being the loss rate became unacceptable. An erradication programme based on serological surveys and slaughter of reactors followed, with the result that today these serotypes are rarely isolated in countries using modern production methods (Stuart, 1984). These specific serotypes rarely infect humans. In contrast, Salmonella food poisoning is caused by the non-host adapted serotypes of which there are ca. 1800. They rarely cause serious economic loss in the poultry industry, any infection being normally sub-clinical. Indeed such serotypes account for less than one per cent of diagnosed poultry diseases (Stevens, 1971). They are nevertheless commonly found in the caeca. They may colonise these blind sacs very soon

after the chickens have hatched (Gustafson and Kobland, 1984) and, through being shed intermittently in the faeces, may be recycled through the birds environment, animals and even eventually man (Williams, 1981a). Medication of an infected flock is not effective and probably prolongs shedding (Stuart, 1984).

Eggs are considered to be germ free in the oviduct and hence at oviposition (Board, 1966). This may be due in part to the honey-combed structure of the immature shell membranes (Plate 13, see also Hoffer, 1971) which probably restrict bacterial penetration should organisms occur in the oviduct. In this respect it is notable that the honey-combed structure persisted until after the cone layer had fused (Plate 15c). This factor may help prevent the contamination of the egg in the oviduct which might otherwise result, for instance, from reverse-peristalsis - contaminated material from the general environment being drawn up into the oviduct. Romanoff and Romanoff (1949) list a range of objects (parasitic worms, insects, gravel, sand, feathers, horse hair, faecal material) that have been found in eggs. They concluded that reverse peristalsis was responsible for such inclusions.

As the vast majority of eggs are contaminated post-oviposition, the recycling of Salmonella serotypes via faecally-contaminated eggs is of particular commercial importance. Unfortunately salmonellosis (other than that due to the host specific serotypes) in poultry is impossible to distinguish from other generalised infections by simple inspection. Any potentially deliterious effect on the growth rate of chicks is compensated in most cases by the growth potential of the birds which have been rigorously selected for high growth rates (Stuart, 1984). This makes any attempt at surveillance time consuming, expensive and of little direct benefit to the farmer.

Human fatalities from non-host adapted serotypes in countries with modern medical care are rare - less than 0.2% of reported cases (Horwitz and Gangarosa, 1976). Thus until recently there was no immediate incentive for producers to clean up their flocks.

Although human salmonellosis outbreaks do not normally give rise to public concern, unless they affect hospitals especially those containing elderly people or cause very large outbreaks, the treatment of such episodes costs the health authorities a considerable sum. It has been estimated, for example, that outbreaks cost over $1,000,000,000 per annum in the United States alone (Todd, 1980). In the UK the Health Economic Research Unit at Aberdeen University is now costing outbreaks of salmonellosis (see Cohen et al., 1983). Indeed such studies are now an important cause of the growing pressure from the public health lobby for poultry producers to take steps to reduce the extent of poultry-related salmonellosis (Williams, 1964, Cohen and Blake, 1977).

At present fresh eggs for the retail market pose no threat as less than 0.2% are likely to contain salmonellae (Baker et al., 1980) and any contaminants are likely to be killed by cooking, providing the temperature exceeds 74°C (D'Aoust and Stotland, 1980). Incubation however provides near optimal conditions for Salmonella growth (Williams, 1978). Because of the scale of modern poultry operations cross-contamination in the hatcher, brooder or rearing house via fluff and faeces is a major problem, often associated with "in house" serotypes (Furuta and Maruyama, 1981, Morgan-Jones, 1982) many of which are known to have been introduced in the feed (Williams, 1981a, b). Salmonella hadar, for example, is regarded as a serotype peculiar to one or two large poultry operations in the UK (Silliker, 1982). Only one contaminated egg needs to hatch for all the chicks/poults to

be put at risk. The experience of the microbiologists of one major British company is notable in that they have found "widespread evidence that egg borne salmonellae remains a problem" yet they have only "succeeded in isolating a Salmonella once from around 10,000 eggs examined.....other commercial hatcheries have had similar experiences" (C. Baxter-Jones, pers comm). Those producers who have an "in house" serotype and who have been constantly nagged by regulatory agents and large customers, have appreciated that "self-discipline" - ie elimination or, at least control, of depots of infection - is preferable to government intervention. In large rearing units cross-contamination however is a problem of such magnitude that it is generally accepted that it is not economically feasible to eliminate a serotype once it has become established in a flock (Stuart, 1984). Therefore control measures must be taken to prevent the newly hatched chicks from becoming contaminated. One approach is to free the eggs set for hatching from contaminants. As such a policy has proved successful in the control of mycoplasmas in turkey flocks (P. Wilding, pers comm), there would seem to be good prospects for salmonellosis control by this approach also. A complimentary and perhaps associated step would be competitive inhibition - the Nurmi effect - in the newly hatched chick. The susceptibility of the young chick (Stuart, 1984) - less than one organism per 10g of feed results in infection - to intestinal colonisation by salmonellae is the result of their simple gut flora in the few days post-hatching. The efficacy of competitive exclusion probably rests in part on the complex flora used to infect the chick (Mead and Impey, 1984). It must be realised, however, that competitive exclusion is not a panacea. Conventional treatments - control of food, fumigation and general hygiene - are also necessary to bring about a decrease in the incidence of salmonella-contaminated

poultry meat (Pivnick and Nurmi, 1982, Schleifer, 1985).

Egg cleaning has always been practiced by table egg producers (Moats, 1978). The methods are often applied without an appreciation of the contribution of the various parts of the shell to an egg's wellbeing. Although the table egg producer may regard the egg's integument as a neat, resilient package for his product, in practice its many and, at first sight, conflicting biological roles may make an egg vulnerable to infection if good washing practices are not observed. The avian egg contains all the nutrients and water required by the developing embryo. There is a need to exchange respiratory gases with the environment and for the egg to lose ca. 16% of its fresh weight as water vapour prior to pipping (Drent, 1975). The shell must therefore be sufficiently porous to allow an adequate exchange of respiratory gases yet not so porous as to cause an unacceptable depletion of the water reserve. Many workers (Walden et al., 1956, Kraft et al., 1958, Brown et al., 1965) have surmised that an egg's vulnerability to infection is associated with a highly porous shell. The present study (Fig. 11) did not support this view thereby supporting the contentions of Board and Halls (1973a) who also found that there was no correlation between shell porosity and water uptake. In that study as well as the current one water uptake was used as an indirect index of a shell's resistance to bacterial penetration.

Although most pores in hen egg shells are "post horn" in shape (Tullett et al., 1975), the diameter of neighbouring pores may vary considerably (Plate 21). Indeed pores of exceptional diameter were found to be particularly vulnerable to flooding (pp 139).

Many eggs are incubated in dry conditions (eg pigeon, dove, ostrich - Board, 1975, Board et al., 1977). These have no cover over

the outer orifices of their pore canals. Other nest environments are wet and muddy yet the majority of the pores must remain open if the exchange of respiratory gases is to continue. A range of pore systems have apparently evolved (Tyler, 1964b, 1965, 1966, 1969b, Tyler and Simkiss, 1959, Board, 1980, Board and Perrott, 1979, 1982 Board et al., 1977, 1984) so that gaseous flux is not impeded during brooding even though the shell's surface is exposed to dirt, preening oils and, of course, microorganisms. Well developed covers (inorganic - either crystalline such as vaterite or amorphous, see Board, 1980, Board et al., 1984) or cuticles (organic) are notable features of such eggs. Evidence of the cuticle on Guinea fowl eggs acting as a filter was presented by Board and Perrott (1982). The hen's egg in nature, which like those of the Guinea fowl is incubated in a scrape in the soil, is also enveloped in a cuticle. Board (1980) noted that the cuticle's "...main contribution appears to be as an impediment to the flooding of the pore canals". This view was expressed earlier by Romanoff (1931) and Vadehra et al. (1970) and discussed by Lack (1968) in the context of birds nesting in wet environments. The present study has confirmed that, of the components in the hen egg's integument, the cuticle is the principal resistance to fluid movement and presumably to bacterial infection also (Fig. 11). This view has not always been entertained. Indeed the authors of earlier reports (Garibaldi and Stokes, 1958, Kraft et al., 1958, Lifshitz et al., 1964, Brown et al., 1965) contended that, when compared with the inner shell membrane, the shell (and by implication the cuticle as they rarely distinguished between the two) made a "relatively small" contribution to the prevention of bacterial contamination.

It needs to be stressed that the studies discussed above have been concerned with eggs of at least 24h of age. In practice this is not a

serious criticism of the work because the washing of table eggs or sanitization of hatching ones is normally done several hours post-laying. My studies have shown that the properties of the cuticle immediately following oviposition change rapidly in respect of its resistance to bacterial penetration. The contribution of the immature cuticle needs to be recognised by the poultry industry so that contamination of the egg particularly with salmonellae in the laying house is recognised generally and steps taken to control it.

There have been vague, almost anectodal, suggestions previously (see Romanoff and Romanoff, 1949) that an egg is most easily infected immediately following oviposition. In the present study of brown eggs it was found that the cuticle's structure changed significantly during the first 30 seconds or so following oviposition. The "wet" cuticle of the freshly oviposited eggs had a very open "frothy" appearance (Plate 2). This changed rapidly as the cuticle dried - the numerous fissures closing up to form solid plaques. Once dry and assumed to be matured, the characteristic microstructure of the brown egg cuticle was apparent, a fissured layer overlaid by dense plaques (Plate 4). The cuticle's structure appeared to be relatively stable thereafter.

When challenged with faecal material the immature cuticle was found to offer significantly less resistance than the mature one to bacterial penetration (Fig. 8). This is probably due to three factors. Firstly the immature cuticle is moist and the water phase is probably ideally suited to the transfer of bacteria from the faeces to the shell membranes via the fluid-filled pore canals. Secondly minimal protection would be offered to the underlying pore canals by the very open and highly fissured structure of the immature cuticle. Thirdly, the egg at oviposition is at body temperature and contact with the cool environment will lead to contraction of the contents and a

negative pressure within the egg. Indeed the air cell is formed at this stage to accommodate changes in the volume of the yolk and white vis à vis the shell (Romanoff and Romanoff, 1949). Thus a situation analogous to the temperature differential dip used in this and many other studies obtains and bacteria may well be sucked in with the moisture in the cuticle. It is interesting to note that the resistance of an immature cuticle was reflected in the mature state also (Fig. 8). This suggests that selection for "good quality" immature cuticles is worthy of the consideration of poultry breeders.

The immature cuticle is so mechanically weak that it is easily damaged. Indeed when an egg rolls down the wire mesh floor of a battery cage scratches in the cuticle leads to permanent damage. This is accentuated if the bird causes the egg to be scuffed against the floor. The mature cuticle is not damaged by such agencies (Simons, 1971). Scratched cuticles were seen on >50% of eggs observed in this study. Excessive stain uptake was commonly associated with the shell membranes underlying a scratched cuticle. Thus there is a need to recognise that damage to the immature cuticle may increase an egg's vulnerability to infection. The damage to the cuticle by uric acid in hens' faeces (Ball et al., 1975) is likely to be another important cause of impairment of the cuticle's efficacy as a barrier to bacterial infection.

My results on the change from immature to the mature state of the cuticle probably aids an interpretation of the observations by Smeltzer et al. (1979). They found that in a deep litter system the bacteria penetrated the shell integument of 10.5% of nest and 15.3% of floor eggs (a statistically significant difference). This difference may reflect, in part, the incidence of eggs with immature cuticles coming into direct contact with moist faecal material.

Indeed Harry (1963) concluded that " ....systems of management in which laying birds are maintained in contact with faecal material would appear to favour shell contamination with potental spoilage organisms". Modern nest box practices may have been adopted perhaps unwittingly to accommodate the vulnerability of the cuticle on the egg at oviposition. For example the addition of formaldehyde prills to nest boxes ensures low levels of contamination and therefore reduced challenge to the eggs at the time of lay (P. Wilding, pers comm). The use of "Astroturf" as a nest liner presumably minimises the area of immature cuticle that makes contact with infected surfaces. In view of my observations on the immature cuticle, nesting boxes ought to be examined with the objective of not only minimising bacterial challenge but also of reducing the incidence of damage to such cuticle.

The evidence discussed above focused attention on the important role of the cuticle in protecting eggs from infection. It can be inferred that cuticle quality may be an important factor also in the control of infection and maybe in the spread of Salmonella. Yet this structure has never been considered in the intensive breeding programmes to which both laying and table birds have been subjected. Such neglect is surprising in view of the observations that brown eggs are more resistant to bacterial penetration than white ones (Walden et al., 1956, Board and Halls, 1973a). This was confirmed in the present study (Fig. 10). As the functional pore area for the brown and white eggs was similar, the differences in water uptake by the two groups was attributed to the properties of the cuticle.

This study has shown also that marked differences exist in the microstructure of the cuticles of white and brown eggs. Micrographs (Plate 3) showed the former to have a delicate open

structure (not unlike that of the immature brown cuticle - Plate 2) while the brown cuticle appeared to be much denser. A difference in microstructure was suggested by the observations of Board and Halls (1973a) who noted that EDTA caused the white cuticle to flake off whereas the brown one was detached as two or three sticky "sheets". These differences were noted in this study also and it was found that the weight of cuticle per unit area of white eggs was 35% less then that on brown ones. Of course a destructive system such as gravimetric assessment of cuticle quality would be an impracticable method in a breeding programme.

Staining cuticles with Edicol Supra Pea Green H revealed further differences between white and brown cuticles. Brown eggs laid by pullets stained deep green whereas those laid by mature hens stained bluey green. White eggs stained blue. These differences were not due solely to the difference in background colour of the shell/cuticle. The mechanism by which Edicol Supra Pea Green H binds to the cuticle is not known. My observations suggest a possible interpretation. Thus it was found that the greater the extent of fissuring - and hence in certain instances the greater the vulnerability of the shell to bacterial penetration (pp 139) - the bluer the cuticle was stained. This was associated with the Green S portion of the whole stain being retained by the heavily fissured cuticle. The other, more water soluble component of the dye, may be differentially removed when the excess stain was washed off. Of course another and, as yet, unidentified cuticle component with an affinity for Tartrazine and an involvement in the process that limits cuticle fissuring - perhaps through imparting elasticity - may be involved. Should such a component deteriorate with time, it could account for changes in stored eggs noted by previous workers (see Simons, 1971), the cuticle

deteriorates or "shrinks" at rates proportional to the storage temperature. Ball et al. (1975) found that refrigerated storage prevented any serious deterioration (measured by staining) of the cuticle for up to 36 days. At ambient temperatures the onset of deterioration was detectable by day 19. The differential uptake by the cuticle of Tartrazine and Green S suggests that Edicol Supra Pea Green H may be used as an indicator of cuticle quality.

Indeed Ball et al. (1975) used this stain for such a purpose. They scored for quality according to stain intensity, irrespective of whether it was blue or green. The present study has shown that the intensity and tint of the stained cuticle are equally important. Staining "for quality" has two disadvantages at present - firstly without fully understanding the reasons for the different shades/tints, it is difficult to appreciate what they signify and secondly very few hen eggs stain evenly so that any measurement of overall cuticle quality is highly subjective. For staining to be commercially effective a fuller understanding of the interaction between stain and cuticle would be required or a better staining system selected.

At present there is not a totally acceptable method for measuring shell quality. Indeed after investigating several alternative methods, staining had to be adopted in this study to assess cuticle quality.

Once there was a reliable commercial method of measuring cuticle quality, an investigation into the genetic control of this attribute could be considered. Board and Halls (1973a) and Ball et al. (1975) discussed the question as to whether the amount and distribution of cuticle on a hen's egg was a characteristic trait or a random event. Ball et al. (1975), who worked with eggs from hens kept on the farms of a large commercial poultry breeder, stained eggs from four pure

and six commercial lines. They observed differences in the cuticles on the eggs from the commercial strains as well as from the pure strains. Indeed all the eggs from one of the pure strains stained blue and between 25 - 67% of the eggs from the commercial strains had cuticles that stained this colour also. Blue staining suggests (judging from the results of my study) a highly fissured, poor quality cuticle. Vadehra et al. (1970) noted a considerable variation in the efficacy of the cuticle on eggs laid by three commercial strains as a barrier to bacterial penetration. These observations can be taken as evidence of genetic influence on cuticle quality.

Does a hen always lay an egg with a consistent cuticle quality? As can be seen from Fig. 5 the cuticles on pullet eggs were invariably of a very high quality, whereas those on hens' eggs (of the same variety) were not. Cuticle quality appears therefore to be related to the age of the laying bird. Indeed the deterioration in cuticle quality appears to occur along with deterioration in other features of the egg viz increases in functional pore (Rahn et al., 1981a, Tullett and Smith, 1983) and in shell surface area (Rahn et al., 1981a). Ball et al. (1975) reported that there was no significant difference between the cuticle quality as judged by staining, on eggs laid by birds during the 22 to 68 weeks of lay. They did note, however, the other changes noted above. The reasons for the discrepancy between their studies and the present one may well be due to the highly subjective interpretation of the staining technique.

The cuticle on pullet eggs was not only of a uniformly high quality but each bird deposited a cuticle of a distinctive nature - identifiable due to the combination of stain intensity and tint. The reasons for these characteristics were not identified when viewed with the SEM. This situation did not obtain with the

cuticle on eggs laid by the mature hens. In this instance cuticle quality was poor and extremely variable. Moreover there was a predominant trend in that the cuticle on the poles of hens' eggs tended to be of a lower quality than that around the shoulder of the egg. Could this be a plausible interpretation of the oft repeated claims that the broad pole is the least effective barrier to bacterial penetration (Romanoff, 1943, Lorenz et al., 1952).

Besides areas of low quality cuticle predisposing the egg to contamination, over-sized or "patent" pore, calcareous lumps and other surface blemishes as well as mechanical weakness leading to shell fracture may negate the cuticle's efficacy. Indeed the use of "Grade cracks" shell eggs by bakeries has been associated with salmonellosis (D'Aoust and Stotland, 1980). Studies by Carter (1977) and Nestor and Bacon (1981) suggest a high heritability for rough shelled or pimpled eggs. Furthermore Lorenz et al. (1952) noted that eggs from a few birds were always contaminated if challenged - suggesting possibly a genetically controlled defect.

Should the egg integument become contaminated with Salmonella in the nest box then the organism may well penetrate to the shell membranes and antimicrobial agents will have to be used to eliminate them if the chick and ultimately the consumer are to be protected. The cuticle's resistance to hydraulic flow has to be overcome by work if antimicrobial agents are to be introduced to the shell membranes. The poultry industry needs, therefore, to consider means of maximising this uptake. Negating the resistance of the integument has been studied by All et al. (1964) and Ekperigin and McCapes (1977). They removed the cuticle by immersing eggs in dilute acids and used the differential temperature dip in a successful attempt to accentuate uptake of antibiotics in hatching eggs in a programme that was

intended to irradicate mycoplasmas. This study has shown that with the temperature differential dip technique, more than half of the final water uptake was achieved within the first five minutes of immersion. This feature does not appear to have been recognised by the poultry industry. In practice it would appear that the thread of fluid connecting the liquid in which the egg is immersed and the shell membrane during the temperature differential dip obtains for this period only. In other words, the pores impose a resistance such that the osmotic uptake of water, which occurs in shell-less eggs, (Board and Halls, 1973a, Sparks and Board, 1984) is prevented.

As the size of the air cell influenced the amount of water taken up by an egg, storage under "dry" conditions before temperature differential dipping may be a useful practice providing the blastoderm was not harmed. Storing eggs for three days at 37.5°C/r.h. 0% resulted in an average intake that was significantly higher than that achieved by removing the cuticle from day-old eggs. Obviously any commercial application is going to be dependent on the effect that the treatment has on embryo viability. These results demonstrate, however, the importance of this hitherto ignored parameter when considering fluid uptake.

Reducing the surface tension of the water is another possible way of increasing water uptake in the initial five minutes of the differential temperature dip. This was achieved in the present study by the use of the surfactant, Triton X-100. A mean increase of 344% over the controls was achieved. As Board and Halls (1973a) failed to accentuate water uptake by treating eggs with Teepol, the choice of surfactant appears to be critical. Not only must it accentuate uptake but it must not impair embryogenesis.

Board and Halls (1973a) suggested that the pressure differential

was satisfied not only by the adsorption of water but also by the adsorption of gases dissolved in the water. Indeed in the present study "degassing" the water caused a significant increase in water uptake (204%). The reasons for this are not clear however. As the volume of gas dissolved in one litre of water is small (ca. 0.5%, Villee et al., 1978), the increased water uptake by the eggs is not due presumably to liquid being taken up in place of the dissolved gas. One may postulate that water in contact with the rough surface of the cuticle and pore canal results in non-uniformity of flow and consequently widely varying pressures within the pore canal. Should the pressure fall to the vapour pressure of water, then the latter would boil with the formation of small bubbles which would subsequently collapse as they reached a point where the pressure was higher than vapour pressure. This is called vapour cavitation - when the liquid has air in solution (as in the dip solution) and the pressure falls, then air cavitation may occur also (Massey, 1984). These bubbles, whether from vapour or air cavitation, could result in air locks and disturbed fluid flow. To avoid such problems the absolute pressure head should be maintained at ca. 20 kPa (Massey, 1984), a situation that was unlikely to have existed in the experiments under discussion.

It is feasible therefore that degassing the water was associated with a reduction in the amount of cavitation within the pore canals and thereby a diminution in the disturbance to the fluid flow through the canals. In the context of egg cleaning as opposed to "sanitisation" artificial aeration of the water might be benificial when microbial penetration of the shell membrane needs to be avoided. The reverse situation obtains if antimicrobial treatment of hatching eggs is being considered.

Temperature of incubation has a marked effect on the progress of

infection of eggs with pure cultures of bacteria (Wolk et al., 1950; Ayres and Taylor, 1956; Board and Ayres, 1965). The last mentioned, for example, found that the size of populations of Serratia marcescens, which remained in the shell membranes following inoculation, did not change with storage at 10°C. Those of Pseudomonas fluorescens increased slowly in the shell membranes during the 14 - 21 day storage period and there was a progressive increase also in the number of pseudomonads in the albumen. As there was no evidence of a union between the yolk and membranes, the authors presumed that contaminants of the albumen were not killed and indeed multiplication occurred and rotting ensued when some reached the surface of the yolk, a phenomenom observed much earlier by Sharp and Whitaker (1927), and were thus able to satisfy their Fe^{3+} requirement (Tranter, 1982). In my study incubation of pure cultures of salmonellae in albumen at 6.5°C (Fig. 30) resulted in little, if any, growth. This is in agreement with the findings of Stokes et al. (1956) who recommended that a storage temperature of $<10^{\circ}C$ be used in order to prevent the growth of salmonellae in shell eggs. Sharp and Whitaker (1927) noted that the efficacy of the albumen's antimicrobial properties was enhanced when the alkalinity of the albumen increased post-oviposition. It was noted in the present study also that the growth of salmonellae in albumen at a pH near to neutral was greater than in that with an alkaline reaction (Fig. 24).

Storing infected eggs at temperatures between 15 and 30°C results in a lag period of 10 - 20 days for the organisms confined to the membranes. This is followed by an abrupt rather than a progressive increase in the number of cfu both within the membranes and in the albumen (Zagaevsky and Lutikova, 1944; Gillespie and Scott, 1950; Bigland and Papas, 1953; Stokes et al., 1956; Orel, 1959; Fromm and

Monroe, 1960; Garibaldi and Bayne, 1960; Brooks, 1960; Board and Ayres, 1965). Board (1964) and Board and Ayres (1965) contended that the lag phase was terminated by the union of the yolk and the infected shell membranes, an event which short circuited the antimicrobial defence of the albumen. Brooks (1960) and Hartung and Stadelman (1963), on the other hand, surmised that a deterioration in the membranes was responsible. Such an interpretation seems improbable in view of the results obtained by Board (1964), furthermore the present SEM studies of shell membranes did not reveal any changes in this structure during incubation. Board (1964) recorded a lag period of the order noted above when the eggs were stored (27°C) with the infected air cell uppermost, the sudden increase in the number of cfu within the membranes being associated with evidence of contact of the yolk and shell membranes and the onset of rotting. Eggs stored with the infected air cell downwards showed only a slow increase in the incidence and extent of contamination of the albumen. Hence it is Board and Fuller's (1974) contention that it is the location of the nidus of infection relative to the movement of the yolk that determines not only the incidence but also the rate of rotting of eggs. The present studies with whole eggs provided evidence of the efficiency of the chemical defence in the albumen. It is noteworthy that with incubation between 15 - 30°C albumen *in vitro* was bactericidal to the salmonellae used in this study (Fig. 30). The efficacy of the albumen's antimicrobial properties varied however with the species. Thus over the range of temperatures (6.5 - 44°C) used in this study, *S. hadar* appeared to be more resistant than the other serotypes to the inimical environment of the albumen. This attribute of *S. hadar* may indicate that it is adapted to egg transfer and hence explain its persistence

in flocks of turkey and its connection with food poisoning outbreaks associated with turkey meat (Silliker, 1982).

At a temperature of 37°C, Board and Ayres (1965) observed a sharp decline in the number of cfu of Serratia marcescens in the shell membranes due, it was claimed, to the migration of organisms to the underlying albumen in which the contaminants tended to die off. Between the 5 - 7 day of incubation the yolk made contact with the infected membranes. This was followed by a rapid multiplication of the organisms both within the membrane and albumen. Thus the available evidence shows that at incubation temperatures >15°C, the onset of heavy contamination of the albumen, and hence of rotting, is associated with the rate of deterioration of the albuminous sac. In other words, these observations indicate the important role of the biological structure in the eggs defence against bacteria. In this study the antibacterial properties of the albumen were enhanced at temperatures of 37.5°C and above (Fig. 30). Thus at egg incubation temperature (37.5°C), the efficacy of the albumen is almost maximal. This feature is not generally recognised in the hatching industry.

Although many studies have been concerned with the ability of single strains to infect eggs, only one - Seviour and Board (1972) - has examined the behaviour of mixed infections. Their work probably simulated the conditions that obtain in eggs infected either in the nest or on the floor of commercial holdings. They found that temperature had an important elective action. At 37°C the coliform organisms achieved dominance within a few days of incubation even though the inoculum was dominated by Gram-positive bacteria at the outset. The authors attributed this effect to the faster growth rate of Gram-negative compared with Gram-positive organisms. As in the studies with pure cultures discussed above, the union of yolk and

membranes initiated a second phase of multiplication. The population which developed in the albumen tended to be dominated by a single species/strain of bacteria whereas several were recovered from the shell membranes of such eggs. It is difficult to compare results from different studies in which mixed infections were used because of marked differences in the composition of the initial inoculum. Indeed Seviour and Board (1972) found that the composition of their inocula (prepared by macerating eggshells) and the course of the infection in eggs inoculated in the air cell varied greatly according to the history of the eggs used for the procurement of the inocula (ie battery produced or free-range). In my study the inoculum was prepared from faecal material taken from caged birds and the eggs were incubated on their sides (and turned around the long axis) - not air cell uppermost as is often the case. Another difference between the two studies is that in the current one the relative humidity of the incubator was maintained at 60%. Nevertheless certain trends were common to both studies. The coliforms (Figs. 21b, 22b), for instance, were the dominant organism throughout the incubation period. The control of the relative humidity at 60% prevented the micrococci (Figs. 21f, 22f) from achieving dominance as they did in Seviour and Board's study when the contents lost excessive water thereby imposing an a_w conducive to the growth of these organisms. Compared with the coliforms (Fig. 22b), the growth of enterococci (Fig. 22c) and pseudomonads (Fig. 22d), was restricted, possibly by an interaction between the incubation temperature and the stress imposed by the environment. The behaviour of the salmonellae deliberately added to a faecal inoculum (Figs. 20d, 21e, 22e) tended to resemble that of the coliforms. This was taken as evidence that, under the conditions pertaining in this study, neither the incubation environment nor the

general contaminants of faeces had an adverse effect on these organisms. This point is of particular relevance to the hatchery manager because it suggests that even one egg with very low numbers of salmonellae could become a major depot of infection in an incubator should it explode as a consequence of rotting or, alternatively, should embryogenesis proceed to completion without the number of these organisms in the shell membranes having diminished.

The delay between infection of the shell membrane (Fig. 23g) and contamination of the albumen was a feature common to my study as well as that of Seviour and Board's (1972) and the many studies in which pure cultures were used. It was notable that increasing the size of the inoculum from ca. 10^3 cfu/membrane to ca. 10^6 cfu/membrane did not influence appreciably this lag period. This feature was noted by Board (1968). Large inocula were found to decrease the time taken by the contaminants to colonise the albumen. Indeed once the inoculum level exceeded 10^6 cfu/membrane (Fig. 20a, e), the lag period was reduced to <2 days. Brooks (1960), Hartung and Stadelman (1963) and Board (1964) have reported that the albumen underlying the membranes was rapidly invaded when large inocula of pure cultures were used to infect the inner shell membrane of the air cell.

Rodgers (1973) reported that inoculum-dependant growth was a feature of some strains of E. coli incubated in human serum in which transferrin was the major cause of growth inhibition. He suggested that large but not small inocula in an alien environment might produce essential metabolites, in this case siderophores that scavenge the Fe^{3+} required for bacterial growth. Such an explanation may account for the inoculum-dependance of the extent of albumen contamination observed in this and other (Lutsky and Bell, 1953; Hartung and Stadelman, 1963; Tranter, 1982) studies with egg albumen. The inoculum level determined

also the rate and extent to which salmonellae colonised the albumen (Fig. 20).

The addition of iron to an inoculum in order to negate the effects of ovotransferrin has been shown to result also in the rapid colonisation of the membranes and albumen (Board, 1964). Furthermore Board *et al.* (1968) noted that the membranes remained stained with the iron salts for at least eight weeks. In the present study, iron salts were shown to bind to the mantle of the membrane fibres (Plate 22) thereby providing a reservoir of this element for microbial contaminants. In my study, in which faecal material was used as an inoculum, the possibility that some iron was carried over from the faeces cannot be ignored especially when the larger inocula were used. This may partially explain the apparent inoculum-dependence of the rate of albumen contamination. In a commercial context, the carry over of iron via faecal material would not presumably be an unusual feature.

Although very high counts of bacteria occurred in the albumen either in the eggs or *in vitro*, there was rarely evidence of rotting in the former case. Indeed of the organisms isolated from the faecal material (Table 4), only *Proteus vulgaris* has been shown to be capable of producing a visible rot in eggs (Board and Board, 1968). It cannot be assumed therefore that candling would identify every egg that is heavily contaminated. Moreover the vast majority of contaminated eggs, when broken open, showed no overt signs of rotting - only a slight turbidity in the albumen. Similar observations were made by Board (1964). This feature raises questions about the use of incubator clears for food purposes.

The course of the bacterial invasion through the shell membranes (Figs. 21, 23) was followed with Transmission Electron Microscopy (Plates 24 - 30), as opposed to Scanning Electron Microscopy which

was used by Tung et al. (1979) in the only other study of this type. There were three reasons for using TEM. Firstly the methods used to prepare sections were considered to be less likely to introduce artefacts than the SEM preparative techniques. Secondly, the TEM offers greater resolution and thirdly, the site to be examined could be selected with great precision.

The survey showed that within the first two days of incubation (Plate 24c) the number of contaminants increased throughout the membrane. Candlish (1972) but not Tung et al. (1979) noted that contaminants tended to accumulate against the limiting membrane. My results are in accord with those of the latter workers. Although there was a ten fold increase in the number of viable organisms (Fig. 21d) during the first few days of incubation this increase would appear to be too small to account for the number of organisms seen in Plate 24c, d. This is taken as circumstantial evidence of a rapid multiplication of the organisms during the initial period of incubation, possibly as a result of iron carry over, followed by a decline in numbers. As the membranes are not bactericidal per se (Stokes and Osborne, 1956; Garibaldi and Stokes, 1958; Board, 1965a), the albumen's ovotransferrin may have been responsible for this decline in numbers.

Contaminants must penetrate the limiting membrane in order to infect and eventually colonise the albumen and ultimately the yolk. The method by which this membrane is penetrated was not identified in this study. Indeed even when the albumen was heavily contaminated there was no evidence of degradation of the limiting membrane. Other authors (Fujii and Tamura, 1970, Simons, 1971, Simons and Wiertz, 1970, McFarland et al., 1971) have reported that a few "holes" occur in the limiting membrane. Considering the nature of the techniques used to study this delicate structure, it is possible that these holes were

artefacts. Candlish (1972) supported the "hole hypothesis" and contended that the scarcity of holes represents the rate-limiting step for microbial penetration of the albumen. Wedral (1971) found no significant difference however between the penetrating ability of proteolytic and non-proteolytic strains of Pseudomonas aeruginosa. Similarly she failed to find a commercial or bacterial enzyme that would enhance penetration. A micrograph taken in this study (Plate 27b) appears to show bacterial degradation of the limiting membrane. However even with SEM - with which relatively large areas can be surveyed - there was no evidence of the limiting membrane being disrupted. During the course of this study it was noted that the albumen of eggs used in water-uptake studies (stained with Edicol Supra Pea Green H) occasionally contained streaks of stain - indicating that the stain had breached the limiting membrane. This may be further evidence in support of Candlish's hypothesis. Whether bacteria penetrate the limiting membrane via "holes", by digesting the limiting membrane or by a combination of the two, the evidence from this and other studies indicates that the infection and subsequent colonisation of the albumen is a gradual process - the abrupt increase in the number of viable contaminants (as seen in Figs. 21g, 22g, 23g) being associated with growth in the albumen. It is interesting to note that as incubation progresses the albumen's antimicrobial efficacy decreases (Figs. 26 - 29). This may well influence, although not determine, the speed at which the albumen is colonised. Lutsky and Bell (1953) and Hartung and Stadelman (1963) noted that there was a significant correlation between the age of the egg and the time taken for the albumen to be infected. The latter authors contended that this was due to the ease with which the contaminants could penetrate the shell membranes. The results of my

study suggest that it is more likely to be the result of a decrease in the antimicrobial efficacy of the albumen and decay of the biological structure of the egg. Board (see Board and Fuller, 1974) noted that he had been unable to establish rot in incubated fertile eggs which had been inoculated in the air cell. This study showed that there was, however, no significant difference between the antimicrobial efficacy of albumen taken from fertile or infertile incubated eggs during the first six days of incubation - a finding that was in accord with the study of Lutsky and Bell (1953).

All the studies discussed above have one feature in common, eggs were deliberately infected and then incubated at one (controlled) temperature. In practice a hatchery egg will be subjected to various temperatures before setting. The present study considered the influence of temperature changes on the infection process. Storing eggs for seven days at 16°C/r.h. 75% (commercially accepted storage conditions - Jones, 1981) resulted in an increase of nearly 10^3 cfu/membrane, yet there was no detectable contamination of the albumen (Fig. 23a, g). The increase in the total number of cfu was due almost entirely to an increase in the number of coliforms (Fig. 23b), the other principal Gram-negative contaminant in the inocula, pseudomonads, appeared not to grow (Fig. 23d). The number of enterococci (Fig. 23c) and salmonellae (Fig. 23e) increased less than 100 fold. These results differ from those obtained by Seviour and Board (1972) who found that the pseudomonads became dominant at incubation temperatures below 30°C. As noted previously the difference may be due to the different source of inocula. The micrographs show that the organisms had reached the limiting membrane within one day of incubation (Plate 28c) at 16°C and by day seven the membranes were very heavily contaminated (Plate 30c). The infection

appeared to follow the pattern predicted from the pure culture studies discussed above - a slow build up within the membranes but no contamination of the albumen. After seven days storage at 16°C, the incubation temperature was increased to 37.5°C/r.h. 60%. This was associated with a rapid increase in the number of cfu both within the membranes and the albumen in which the numbers rose by > 10^8 cfu/ml (Fig. 23g). The development of salmonellae at the high incubation temperature (Fig. 23e) reflected the trend shown by the total count (Fig. 23a) - the number of cfu increasing by >10^2/membrane after only two days.

The results from this study indicate that, from a bacteriological standpoint, eggs should not be stored prior to being set for hatching. They also pose questions about the time at which a germicidal treatment to eliminate salmonella in the shell membranes should be carried out. In a commercial operation other factors in addition to time relating to bacterial contaminants will have to be considered. For example any recommendations based on microbiological considerations would have to take into account the facts affecting for example the view entertained by many that the rate of hatching is improved by storing eggs for up to three days before setting (Jones, 1981). Should it be found necessary to store eggs, then it would seem sensible to "sanatise" the eggs so that the integument was freed of contaminants.

REFERENCES

REFERENCES

Ackerman, R.A. and Rahn, H. (1980). In vivo O_2 and water vapour permeability of the hen's eggshell during early development. Respiration Physiology 45:1-8.

Alder, H.E. (1965). Salmonella in eggs - an appraisal. Food Technology 623:191-192.

Aisen, P., Leibman, A. and Reich, H.A. (1966). Studies on the binding of iron to transferrin and conalbumen. Journal of Biological Chemistry 241:1666-1671.

Aisen, P. and Listowsky, I. (1980). Iron transport and storage proteins. Annual Review of Biochemistry 49:357-393.

Alls, A.A., Benton, N.J., Kraus, W.C. and Cover, M.S. (1963). The mechanics of treating hatching eggs for disease prevention. Avian Diseases 7:89-97.

Alls, A.A., Cover, M.S., Benton, N.J. and Kraus, W.C. (1964). Treatment of hatching eggs for disease prevention - factors affecting permeability and a visual detection of drug absorption. Avian Diseases 8:245-256.

Almquist, H.J. and Holst, W.F. (1931). Variability of shell porosity

in the hen's egg. Hilgardia 6:61-72.

Alsaadi, B.M., Williams, R.J.P. and Woodworth, R.C. (1981). A PMR study of the effects of pH and anion and metal ion binding of the histidyl residues of ovotransferrin. Inorganic Biochemistry 15(1):1-10.

Anon. (1978). Cost analysis of an outbreak of salmonellosis - Quebec. Canada Diseases Weekly Report 4(42):165-167.

Anon. (1981). Poultry meat output to recover in 1982. Meat 54(12):9.

Anon. (1982a). Food poisoning and salmonellosis surveillance in England and Wales 1981. British Medical Journal 285 (6348):1127-1128.

Anon. (1982b). Salmonella outbreak "under control". Health and Social Services Journal 92 (4815):1127.

Anon. (1982c). A manual of recommended methods for the microbiological examination of poultry and poultry products. Eds. R.T. Parry, L. Hayson, N.L. Thomas and R. Davis, British Poultry Meat Association.

Ar, A., Paganelli, C.V., Reeves, R.B., Green, D.G. and Rahn, H. (1974). The avian egg: Water vapour conductance, shell thickness and functional pore area. Condor 76:153-158.

Araki, Y., Nakatani, T., Nakayama, K. and Ito, E. (1972). Occurrence of N-nonsubstituted glucosamine residues in peptidoglycan of lysozyme-resistant cell walls from Bacillus cereus. Journal of Biological Chemistry 247:6312-6322.

Arnheim, N. (1974). Multiple genes for lysozyme. In Lysozyme. Eds. E.F. Osserman, R.E. Canfield and S. Beychok, pp 153-161. New York: Academic Press.

Arnold, R.R., Brewer, M. and Gauthier, J.J. (1980). Bactericidal activity of human lactotransferrin. Infection and Immunity 28(3):893-898.

Arnon, R., Teicher, E. and Scheraga, H.A. (1974). Correlation of conformation and biological activity in lysozyme "loop" homologs. Journal of Molecular Biology 90:403-407.

Ayres, J.C. and Taylor, B. (1956). Effect of temperature on microbial proliferation in shell eggs. Applied Microbiology 4:355-359.

Azari, P. and Feeney, R.E. (1958). Resistance of metal complexes of conalbumen and transferrin to proteolysis and to thermal denaturation. Journal of Biological Chemistry 232:293-302.

Azari, P. and Feeney, R.E. (1961). The resistances of conalbumen and its iron complex to physical and chemical treatments. Archives of Biochemistry and Biophysics 92:44-53.

Baker, C.M.A. (1968). Proteins of egg white. In Egg Quality: A Study of the Hen's Egg. Ed. T.C. Carter, pp 67-108. Edinburgh:Oliver and Boyd.

Baker, J.R. and Balch, D.A. (1962). A study of the inorganic material of hen's egg-shell. Biochemical Journal 82:352-361.

Baker, R.C., Goff, J.P. and Timoney, J.F. (1980). Prevelance of salmonellae on eggs from poultry farms in New York state. Poultry Science 59(2):289-292.

Balch, D.A. and Cooke, A. (1970). A study of the composition of hen's egg-shell membranes. Annales de biologie animale, biochimie et biophysique 10(2):13-25.

Balch, D.A. and Tyler, C. (1964). Variation in some shell membrane characteristics over different parts of the same shell. British Poultry Science 5:201-215.

Ball, R.F., Logan, V. and Hill, J.F. (1975). Factors affecting the cuticle of the egg as measured by the intensity of staining. Poultry Science 54:1479-1484.

Baltimore, R.S., Shedd, D.G. and Pearson, H.A. (1982). Effect of iron saturation on the bacteriostasis of human serum: In vivo does not correlate with in vitro saturation. Journal of Paediatrics

101(4):519-523.

Banwart, G.J. and Ayres, J.C. (1957). The effect of pH on the growth of salmonella and functional properties of egg white. Food Technology 11(4):244-246.

Barnes, E.M. and Corry, E.L. (1969). Microbial floral of raw and pasteurised egg albumen. Journal of Applied Bacteriology 32:193-205.

Bates, G.W., Workman, E.F. and Schlabach, M.R. (1973). Does transferrin exhibit ferroxidase activity? Biochemical and Biophysical Research Communications 50:84-90.

Baudrimont, A. and St. Ange, M. (1847). Recherches sur les phenomenes chimique de l'evolution embryonnaire des oiseaux et des bactraciens. Annales de chimie physique, series III 21:195-295.

Baumgartner, S., Brown, D.J., Salevsky, E. Jr and Leach, R.M. Jr (1978). Copper deficiency in the laying hen. Journal of Nutrition 108:804-811.

Bean, K.C. and McLaury, D.W. (1959). The bacterial contamination of hatching eggs and methods for its control. Poultry Science 38:693-698.

Becking, J.H. (1975). The ultrastructure of the avian eggshell. The

Ibis 117(2):143-151.

Bellairs, R. and Boyde, A. (1969). Scanning electron microscopy of the shell membranes of the hen's egg. Zeitschrift fur Zellforschung und mikroskopische Anatomie 96:237-249.

Berger, L.E. and Weiser, R.S. (1957). The beta-glucosaminidase activity of egg white lysozyme. Biochimica et biophysica acta 26:517-521.

Bigland, C.H. and Papas, G. (1953). Experiment in egg penetration by salmonella. Canadian Journal of Comparative Medicine (and Veterinary Science) 17:105-109.

Board, P.A. and Board, R.G. (1967). A method of studying bacterial penetration of the shell of the hen's egg. Laboratory Practice 16(29):471-472,482.

Board, P.A. and Board, R.G. (1968). A diagnostic key for identifying organisms recovered from rotten eggs. British Poultry Science 9(1):111-120.

Board, P.A., Henden, L.P. and Board, R.G. (1968). The influence of iron on the course of bacterial infection of the hen's egg. British Poultry Science 9(2):211-215.

Board, R.G. (1964). The growth of Gram-negative bacteria in the hen's

egg. Journal of Applied Bacteriology 27:(2)350-364.

Board, R.G. (1965a). Bacterial growth on and penetration of the shell membranes of the hens egg. Journal of Applied Bacteriology 28:197-205.

Board, R,G. (1965b). The properties and classification of the predominant bacteria in rotten eggs. Journal of Applied Bacteriology 28:437-453.

Board, R.G. (1966). Review article: The course of microbial infection of the hen's egg. Journal of Applied Bacteriology 29:319-341.

Board, R.G. (1968). Microbiology of the egg (A Review). In Egg Quality: A Study of the Hen's Egg, Ed. T.C. Carter, pp 133-162. Edinburgh: Oliver and Boyd.

Board, R.G. (1975). The microstructure of the cuticle-less shell of the eggs of the domestic hen. British Poultry Science 16:89-91.

Board, R.G. (1980). The avian eggshell - a resistance network. Journal of Applied Bacteriology 48:303-313.

Board, R.G. (1982). Properties of avian eggshells and their adaptive value. Biological Reviews 57:1-28.

Board, R.G., Ayres, J.C., Kraft, A.A. and Forsythe, R.H. (1964). The

microbiological contamination of eggshells and egg packing materials. Poultry Science 43:584-594.

Board, R.G. and Ayres, J.C. (1965). Influence of temperature on bacterial infection in the hen's egg. Applied Microbiology 13(3):358-364.

Board, R.G. and Fuller, R. (1974). Non-specific antimicrobial defences of the avian egg, embryo and neonate. Biological Reviews 49:15-49.

Board, R.G. and Halls, N.A. (1973a). The cuticle: A barrier to liquid and particle penetration of the shell of the hen's egg. British Poultry Science 16:69-91.

Board, R.G. and Halls, N.A. (1973b). Water uptake by eggs of Mallard and Guinea fowl. British Poultry Science 14:311-314.

Board, R.G. and Halls, N.A. (1973c). The effect of iron on the growth of Escherichia coli in albumen taken from the hen's egg. British Poultry Science 14:359-371.

Board, R.G. and Hornsey, D.J. (1978). Plasma and egg white proteins. In Chemical Zoology vol 10 Eds M.Florkin and B.J. Scheer, pp 37-74. London: Academic Press.

Board, R.G. and Perrott, H.R. (1979). The plugged pores of the

Tinamou (Tinamidae) and Jacana (Jacanidae) eggshells. The Ibis 121:469-474.

Board, R.G. and Perrott, H.R. (1982). The fine structure of the outer surface of the incubated eggshells of the hatched Guinea fowl (Numidia meleagris). Journal of Zoology (Lond.) 196:445-451.

Board, R.G., Perrott, H.R., Love, G. and Scott, V.D. (1984). The phosphate rich cover on the eggshells of grebes (Aves: Podicipitiformes). Journal of Zoology (Lond.) 203:329-343.

Board, R.G., Tullett, S.G. and Perrott, H.R. (1977). An arbitrary classification of pore systems in avian eggshells. Journal of Zoology (Lond) 182:251-265.

Boas, M.A. (1927). The effect of dessication upon the nutritive properties of egg white. Biochemical Journal 21:712-724.

Brant, A.W. and Starr, P.B. (1962). Some physical factors related to egg spoilage. Poultry Science 41:1468-1473.

Brooks, J. (1960). Mechanism of the multiplication of Pseudomonas in the hen's egg. Journal of Applied Bacteriology 23(3):499-509.

Brooks, J. and Hale, H.P. (1959). The mechanical properties of the thick white of the hen's egg. Biochimica et biophysica acta 32:237-250.

Brooks, J. and Taylor, D.I. (1955). Eggs and egg products. Report of the Food Investigation Board no. 60. HMSO, London.

Brown, J.R. (1964). Disulphide bridges of lysozyme. Biochemical Journal 92:13p.

Brown, W.E., Baker, R.C. and Naylor, N.B. (1965). The role of the inner shell membrane in bacterial penetration of chicken eggs. Poultry Science 44:1323-1327.

Brown, W.E., Baker, R.C. and Naylor, N.B. (1966). The microbiology of cracked eggs. Poultry Science 45:284-287.

Brown-Mason, A. and Woodworth, R.C. (1984). Physiological levels of binding and iron donation by complementary half-molecules of ovotransferrin to transferrin receptors of chick reticulocytes. Journal of Biological Chemistry 259(3):1866-1873.

Bryant, R.L. and Sharp, P.F. (1934). Effect of washing on the keeping quality of hen eggs. Journal of Agricultural Research 48:67-89.

Buss, E.G. (1969). Genetic interference in the egg transfer, utilisation and requirement of riboflavin by the avian embryo. In The Fertility and Hatchability of the Hen's Egg Eds. T.C. Carter and B.M. Freeman, pp 109-116. Edinburgh:Oliver and Boyd.

Calvery, H.O. (1933). Some analyses of egg-shell keratin. Journal of Biological Chemistry 100:183-186.

Candlish, J.K. (1972). The role of the shell membranes in the functional integrity of the egg. In Egg Formation and Production Eds B.M. Freeman and P.E.Lake, pp 87-106. Edinburgh: British Poultry Science Ltd.

Canfield, R.E. (1963). The amino acid sequence of hens egg white lysozyme. Journal of Biological Chemistry 238:2698-2707.

Canfield, R.E. and McMurray, S. (1967). Purification and characterisation of a lysozyme from a goose egg white. Biochemical and Biophysical Research Communications 26:38-42.

Carter, T.C. (1977). The hens' egg: Incidence and inheritance of some egg abnormalities. Brtish Poultry Science 18:309-313.

Chain, B.M. and Anderson, R.S. (1983). Observations on the cytochemistry of the hemocytes of an insect, Galleria mellona. Journal of Histochemistry and Cytochemistry 31(5):601-608.

Chaiet, L., Miller, T.F. and Wolf, F.J. (1963). Antibiotic MSD-235 II. Separation and purification of synergistic components. Antimicrobial Agents and Chemotherapy 3:28-32.

Chang, K.Y. and Carr, C.W. (1971). Studies on the structure and

function of lysozyme; 1. The effect of pH and cation concentration on lysozyme activity. Biochimica et biophysica acta 229:496-503.

Charlemagne, D. and Jolles, P. (1967). La specificite de divers lysozymes vis-a-vis de substrats de foible poids moleculaire provenant de la chitine. Bulletin de la Societe de Chimie Biologique 49:1103-1113.

Clagett, C.O. (1971). Genetic control of the riboflavin carrier protein. Federation Proceedings 30:127-129.

Clark, J.R., Osuga, D.T. and Feeney, R.E. (1963). Comparison of avian egg white conalbumens. Journal of Biological Chemistry 238:3621-3631.

Cohen, M.L. and Blake, P.A. (1977). Trends in food-borne salmonellosis outbreaks: 1963-1975. Journal of Food Protection 40:798-800.

Cohen, M.L., Porter, I.A., Reid, T.M.S., Sharp, J.C.M., Forbes, G.I. and Paterson, G.M. (1983). A cost benifit study of milk-borne salmonellosis. Journal of Hygiene 91(1):17-24.

Colacino, J.M., Hamel, P.B., Rahn, H., Board, R.G. and Sparks, N.H.C. (1985). The vaterite cover on the eggs of Anhinga anhinga and its effect on gas conductance. Journal of Zoology (Lond) 205(3):425-434.

Conrad, R.M. and Scott, H.M. (1938). The formation of the egg of the

domestic fowl. Physiological Reviews 18(4):481-494.

Cooke, A.S. and Balch, D.A. (1970). Studies of the membrane, mammillary cores and cuticle of the hen egg shell. British Poultry Science 11:345-352.

Cox, G.B., Gibson, F., Luke, R.K.J., Newton, N.A., O'Brien, I.G. and Rosenberg, H. (1970). Mutations affecting iron transport in Escherichia coli. Journal of Bacteriology 104:219-226.

Crombie, G., Snider, R., Faris, B. and Franzblau, C. (1981). Lysine-derived cross-links in the egg shell membrane. Biochimica et biophysica acta 640:365-367.

D'Aoust, J.Y. and Stotland, P. (1980). Salmonella in "Grade cracks" shell eggs. Canadian Institute of Food Science and Technology 13(4):184-187.

DeLange, R.J. and Huang, T.S. (1971). Egg white avidin - III. Sequence of the 78-residue middle cyanogen bromide peptide. Complete amino acid sequence of the protein subunit. Journal of Biological Chemistry 246:698-709.

Dianoux, A.C. and Jolles, P. (1967). Etude d'un lysozyme paurve en cystine et en tryptophane: Le lysozyme de blanc d'oeuf d'oie. Biochimica et biophysica acta 133:472-479.

Donovan, J.W., Bearslee, R.A. and Ross, K.D. (1976). Formation of monoferric ovotransferrin in the presence of chelates. Biochemical Journal 153:631-639.

Doyle, M.P. (1984). Associatuon of Campylobacter jejuni with laying hens and eggs. Applied and Environmental Microbiology 47(3):533-536.

Draper, M.H., Davidson, M.F., Wyburn, G.M. and Johnston, H.S. (1972). The fine structure of the fibrous membrane forming region of the isthmus of the oviduct of Gallus domesticus. Quarterly Journal of Experimental Physiology 57:297-309.

Drent, R. (1975). Incubation in Avian Biology vol 5. Eds D.S. Farmer and J.R. King, pp 333-420. New York: Academic Press.

Edson, R.K.(1980). Mycoplasma meleagridis infection in turkeys: Motivation, methods and predictive tools for eradication. PhD Thesis, University of California, Davis.

Ekperigin, H.E. and McCapes, R.H. (1977). Pre-incubation dipping of turkey hatching eggs, Part 1. Effect of shell treatment on amount and variability of fluid intake. Avian Diseases 21(4):596-604.

Ewing, W.H. (1958). The nomenclature and taxonomy of the Proteus and Providence groups. International Bulletin of Bacteriological Nomenclature and Taxonomy 8(1):17-22.

Ewing, W.H. (1962). The tribe Proteae: its nomenclature and taxonomy. International Bulletin of Bacteriological Nomenclature and Taxonomy 12:93-102.

Farrell, H.M., Mallette, M.F., Buss, E.G. and Clagett, C.O. (1969). The nature of the biochemical lesion in avian renal riboflavinuria III. The isolation and characterisation of the riboflavin-binding protein from egg albumen. Biochimica et biophysica acta 194:433-442.

Farrell, H.M., Buss, E.G. and Clagett, C.O. (1970). The nature of the biochemical lesion in avian renal riboflavinuria V. The elucidation of riboflavin transport in the laying hen. International Journal of Biochemistry 1:168.

Feeney, R.E., Abplanalp, H., Clary, J.J., Edwards, D.L. and Clark, J.R. (1963). A genetically varying minor protein constituent of chicken egg white. Journal of Biological Chemistry 238:1732-1736.

Feeney, R.E., Allinson, R.G., Osuga, D.T., Bigler, J.C. and Miller H.T. (1968). Biochemistry of the Adelie penguin: studies on egg and blood serum proteins. In Antarctic Research Series vol 12 Ed. O.L. Austin, pp 151-165. American Geophysics Union.

Feeney, R.E. and Allinson, R.G. (1969). Evolutionary Biochemistry of Proteins. New York:Wiley (Interscience).

Feeney, R.E., Anderson, J.S., Azari, P.R., Bennett, N. and Rhodes, M.B. (1960). The comparative biochemistry of avian egg white proteins. Journal of Biological Chemistry 235:2307-2311.

Feeney, R.E. and Nagy, D.A. (1952). The antibacterial activity of the egg white protein conalbumen. Journal of Bacteriology 64:629-643.

Fleming, A. (1922). On a remarkable bacteriolytic element found in tissues and secretions. Proceedings of the Royal Society London series B 93:306-317.

Fischer, H. and Kögl, F. (1923). Zur kenntnis der naturlichen Porphyrine. IV Uber das Ooporphyrin. Hoppe-Seyler's Zeitschrift fur physiologische Chemie 131:241-261.

Fischer, H. and Kögl, F. (1924). Zur kenntnis der naturlichen Porphyrine. IX. Uber Ooporphyrin aus Kiebitzeierschalen und seine Beziehungen zum Bluttfarbstaff. Hoppe-Seyler's Zeitschrift fur physiologische Chemie 138:262-275.

Fischer, H. and Müller, R. (1925). Zur kenntnis der naturlichen Porphyrine. XIII. Mitteilung. Uberfuhrung von Hamoporphyrin in Mesoporphyrin und dessen Abbau zum Atioporphyrin. Hoppe-Seyler's Zeitschrift fur physiologische Chemie 142:120-140.

Forsdyke, J. (1981). A simple apparatus for staining and washing a batch of grids. Journal of Microscopy 117(3):437-440.

Fossum, K. and Whitaker, J.R. (1968). Ficin and papain inhibitor from chicken egg white. Archives of Biochemistry and Biophysics 125:367-375.

Fraps, R.M., Hertz, R. and Sebrell, W.H. (1943). Relationship between ovarian function and avidin content in the oviduct of the hen. Proceedings of the Society for Experimental Biology and Medicine 52:140-142.

Frelinger, J.A. (1970). Maternally derived transferrin in pigeon squabs. Nature 171:1260-1261.

Froix, M.F., Pochan, J., Goedde, A. and Hinman, D.L. (1977). A dynamic characterisation via relaxation techniques of the shell membranes of the domesticated fowl (Gallus/gallus (L.)). Polymer 18:1213-1218.

Fromm, D. and Monroe, R.J. (1960). Interior physical quality and bacterial contamination of market eggs as influenced by egg shell permeability. Food Technology 14:401-403.

Fujii, S. (1974). Further morphological studies on the formation and structure of hen's egg shell by scanning electron microscopy. Journal of the Faculty of Fisheries and Animal Husbandry, Hiroshima University 13(1):29-56.

Fujii, S. and Tamura, T. (1970). Scanning electron microscopy of shell formation in hen's eggs. Journal of the Faculty of Fisheries and Animal Husbandry, Hiroshima University 9(1):65-81.

Fukamizo, T., Torikato, T., Nagayama, T., Minematsu, T. and Hayashi, K. (1983). Enzymatic activity of avian egg lysozymes. Journal of Biochemistry 94 (1):115-122.

Furuta, K. and Maruyama, S. (1981). Bacterial contamination on eggs during incubation and hatching and of fluffs of newly hatched chicks. British Poultry Science 22:247-254.

Furuta, K. and Sato, S. (1977a). Studies on the disinfection of hatching eggs: II. The effect of Formaldehyde fumigation on bacteria contaminating the egg shell surface. Japanese Poultry Science. 14:27-32.

Furuta, K. and Sato, S. (1977b). Studies on the disinfection of hatching eggs: I The effect of disinfection by disinfectant solutions on bacteria contaminating the egg shell surface. Japanese Poultry Science 14:223-228.

Gaber, B.P. and Aisen, P. (1970). Is divalent iron bound to transferrin? Biochimica et biophysica acta 221:228-233.

Garibaldi, J.A. (1972). Influence of temperature on the biosynthesis of iron transport compounds by Salmonella typhimurium. Journal of

Bacteriology 110:262-265.

Garibaldi, J.A. and Bayne, H.G. (1960). The effect of iron on the Pseudomonas spoilage of experimentally infected shell eggs. Poultry Science 39:1517-1520.

Garibaldi, J.A. and Bayne, H.G. (1962a). The effect of iron on the Pseudomonas spoilage of farm washed eggs. Poultry Science 41:850-853.

Garibaldi, J.A. and Bayne, H.G. (1962b). Iron and the bacterial spoilage of shell eggs. Journal of Food Science 27:57-59.

Garibaldi, J.A. and Stokes, J.L. (1958). Protective role of shell membranes in bacterial spoilage of eggs. Food Research 23:283-290.

Gavilanes, J.G., De Buitrago, G.G., Martinez del Pozo, A., Perez-Castells, R. and Rodriguez, R. (1982). Pigeon egg white lysozyme: Purification, structural and enzyme characterisation. International Journal of Protein Research 20(3):328-345.

Gillespie, J.H. and Scott, W.J. (1950). Studies in the preservation of shell eggs IV. Experiments on the mode of infection by bacteria. Australian Journal of Applied Sciences 1:514-530.

Green, N.M. (1975). Avidin. Advances in Protein Chemistry 29:85-133.

Gustafson, R.H. and Kobland, J.D. (1984). Factors influencing

Salmonella shedding in broiler chickens. Journal of Hygiene (Camb.) 92:385-394.

Gyorgy, P. and Rose, C.S. (1943). The liberation of biotin from the avidin-biotin complex. Proceedings of the Society for Experimental Biology and Medicine. 53:55-57.

Haines, R.B. (1939). Microbiology in the preservation of the hens' egg. Report of the Food Investigation Board no. 47 HMSO.

Haines, R.B. and Moran, T. (1940). Porosity of, and bacterial invasion through, the shell of the hens egg. Journal of Hygiene (Camb.) 37:453-461.

Halkett, J.A.E., Peters, T. and Ross, J.F. (1957). Studies on the deposition and nature of egg yolk iron. Journal of Biological Chemistry 231:187-199.

Harris, E.D., Blount, J.E. and Leach, R.M. (1980). Localisation of lysyl oxidase in the hen oviduct - implications in egg shell membrane formation and composition. Science 208:55-56.

Harris, W.R. (1983). Thermodynamic binding constants of the zinc-human serum transferrin complex. Biochemistry 22(16):3920-3926.

Harry, E.G. (1963). The relationship between egg spoilage and the environment of the egg when laid. British Poultry Science 4:91-100.

Hartung, T.E. and Stadelman, W.J. (1963). Pseudomonas fluorescens penetration of egg shell membranes as influenced by shell porosity, age of egg and degree of bacterial challenge. Poultry Science 42:147-150.

Hayakawa, S., Kondo, H., Nakamura, R. and Sato, Y. (1983). Effect of beta-ovomucin on the solubility of alpha-ovomucin and further inspection of the ovomucin complex in thick egg white. Agricultural and Biological Chemistry 47(4):815-820.

Healy, D.J. and Peter, A.M. (1925). The relationship of the hydrogen ion concentration of egg white to its germicidal action. American Journal of Physiology 74:363-368.

Heinman, V. (1935). The relative vitamen G content of dried whey and dried skim milk. Poultry Science 14:137-147.

Hertz, R. and Sebrell, W.H. (1942). Occurrence of avidin in the oviduct and secretions of the genital tract of several species. Science 96:257.

Hindenburg, A., Spitzragel, J. and Arnheim, N. (1974). Isozymes of lysozymes in leucocytes and egg white: Evidence for the species-specific control of egg white lysozyme synthesis. Proceedings of the National Academy of Sciences USA 71:1653-1657.

Hiromitsu, W., Yokomizo, H., Wako, H., Isogai, Y., Kosuge, K. and Saito, N. (1984). Unfolding of tertiary structures of proteins. International Journal of Peptide and Protein Research 23(6):657-670.

Hoffer, A.P. (1971). The ultrastructure and cytochemistry of the shell membrane-secreting region of the Japanese quail oviduct. American Journal of Anatomy 131:253-266.

Horwitz, M.A. and Gangarosa, E.J. (1976). Food-borne disease outbreaks traced to poultry, America 1966 - 1974. Journal of Milk and Food Technology 39(12):859-863.

Hoyt, D.F., Board, R.G., Rahn, H. and Paganelli, C.V. (1979). The eggs of the Anatidae: Conductance, pore structure and metabolism. Physiological Zoology 52:438-450.

Ibrahini, I.M., Prager, E.M., White, T.J. and Wilson, A.C. (1979). Amino acid sequence of California quail lysozyme. Effect of evolutionary substitutions on the antigenic structure of lysozyme. Biochemistry 18:2736-2744.

Jolles, P. (1969). Angewantde Chemie int Edn 8:227.

Jolles, P., Javregui-Adell, J., Bernier, I. and Jolles, P. (1963). La structure chemique du lysozyme de blanc d'oeuf de poule: Etude detaillee. Biochimica et biophysica acta 78:668-689.

Jones, D.R. (1981). Quality in hatching eggs and day old chicks. Poultry Section (B) Quarterly Journal ADAS no 139:10-21.

Kampelmacher, E.H. (1983). Irradiation for control of Salmonella and other pathogens in poultry and fresh meats. Food Technology 37(4):117-144.

Kato, A., Imoto, T. and Yagishita, K. (1975). The binding groups in ovomucin-lysozyme interaction. Agricultural and Biological Chemistry 89:541-544.

Kayar, S.R., Snyder, G.K., Birchard, G.F. and Black, C.P. (1981). Oxygen permeability of the shell and membranes of chicken egs during development. Respiration Physiology 46:209-221.

Khoury-Doughly, E. and Spencer, J.V. (1976). The effect of egg albumen on the iron requirement of Salmonella oranienburg. Poultry Science 55(3):1124-1126.

Kierek-Jaszczuk, D. (1981). An atypical form of transferrin phenotypes in the yolk of hens egg. Proceedings Zootechnology 0(26):19-24.

Knothe, H. (1984). Epidemiological aspects of salmonella in man. Journal of the Science of Food and Agriculture 35:639.

Korotkova, G.P. (1957). Antibiotic properties of chicken egg albumen.

Journal of General Chemistry (USSR) 17:275-285.

Kraft, A.A., McNally, E.G. and Brant, A.W. (1958). Shell quality and bacterial infection of shell eggs. Poultry Science 37:638-643.

Krampitz, G. and Witt, W. (1979). Biochemical Aspects of Biomineralisation. Topics in Current Chemistry 59-144.

Kutchai, H.and Steen, J.B. (1971). Permeability of the shell and shell membranes of hens' eggs during development. Respiration Physiology 11:265-278.

Lack, D. (1968). Ecological Adaptions for Breeding in Birds. London: Methuen.

Laschtschenko, P. (1909). Uber die Kiemtotende und entwicklunaphemmende Wirkung huhnere Weiss. Zeitschrift fur Hygiene und Infektionskrankheiten 64:419-427.

Laskowski, M. and Laskowki, M. Jr (1954). Naturally occuring trypsin inhibitors. Advances in Protein Chemistry 9:203-242.

Leach, J.R. Jr (1982). Biochemistry of the organic matrix of the eggshell. Poultry Science 61:2040-2047.

Leach, R.M., Rucker, R.B. and Van Dyke, G.P. (1981). Eggshell membrane protein: A non-elastin desmosine/isodesmosine-containimg

protein. Archives of Biochemistry and Biophysics 207:353-359.

Lee, J.A. (1973). Salmonellae in poultry in Great Britain. In The Microbiological Safety of Food (Proceedings 8th International Symposium on Food Microbiology, Reading England 1972). Ed B.C. Hobbs and J.H.B. Christian, p 197. New York: Academic Press.

Lifschitz, A., Baker, R.C. and Naylor, H.B. (1964). The relative importance of chicken egg exterior structures in resisting bacterial penetration. Journal of Food Science 29:94-99.

Lineweaver, H. and Murray, C.W. (1947). Identification of the trypsin inhibitor of egg white with ovomucoid. Journal of Biological Chemistry 171:565-581.

Lomholt, J.P. (1976). The development of the oxygen permeability of the avian egg shell and its membranes during incubation. Journal of Experimental Zoology 198(2):177-184.

Lorenz, F.W., Starr, P.B., Starr, M.P. and Ogasawara, F.X. (1952). The development Pseudomonas spoilage in egg shells. 1. Penetration through the shell. Food Research 17:351-360.

Lush, I.E. (1961). Genetic polymorphism in the egg albumen proteins of he domestic fowl. Nature (Lond) 189:981-984.

Lutsky, I.I. and Bell, A.E. (1953). Antibacterial action of egg

albumen in embryonic immunity. Poultry Science 32:285-293.

MacGillivray, R.T., Mendez, E., Shewale, J.G., Shina, S.K., Linebeck-Zins, J. and Bren, K. (1983). Primary structure of human serum transferrin: Structures of 7 cyanogen bromide fragments and the assembly of the complete structure. Journal of Biological Chemistry 258(6):3545-3553.

Mandella, R.D., Meslar, H.W. and White, H.B. (1978). Relationship between biotin-binding proteins from chicken plasma and egg yolk. Biochemical Journal 175:629-633.

Manwell, C. and Baker, C.M.A. (1973). Low levels of lysozyme in egg white from the Fairy penguin. The Ibis 115:586-589.

Marshall, M.E. and Deutsch, H.F. (1951). Distribution of egg white proteins in chicken blood serum and egg yolk. Journal of Biological Chemistry 189:1-10.

Marthedal, H.E. (1973). The occurrence of salmonellosis in poultry in Denmark 1935 - 1971 and the eradication programme established. In The Microbiological Safety of Food (Proceedings 8th International Symposium on Food Microbiology, Reading England 1972). Ed B.C. Hobbs and J.H.B. Christian, p 211. New York: Academic Press.

Massey, B.S. (1984). Mechanics of Fluid. 5th Edit. Van Nostrand Reinhold (UK) Co Ltd.

Masshoff, W. and Stolpmann, H.J. (1961). Light and Electron microscopic studies of the shell membrane and calcareous shell of the hen's egg. Zeitschrift fur Zellforschung und mikroskopische Anatomie 55(6):818-832.

Matsushima, K. (1958). An undescribed trypsin inhibitor in egg white. Science 127:1178-1179.

Maw, A.J.G. (1954). Inherited riboflavin deficiency in chicken eggs. Poultry Science 33:216-217.

Maurer, O. (1911). Bacteriological studies on eggs. Kansas University Agricultural Experimental Station Bulletin 180.

McCallion, D.J. (1953). A cytological and cytochemical study of the shell gland of the domestic hen. Canadian Journal of Zoology 31:577-589.

McFarland, L.Z., Garrett, R.L. and Nowell, J.A. (1971). Normal eggshells and thin eggshells caused by organochlorine insecticides viewed by the scanning electron microscope. Proceedings of the 4th Annual Scanning Electron Microscope Symposium, Chicago.

Mead, G.C. and Adams, B.W. (1977). A selective media for the rapid isolation of pseudomonads associated with poultry meat spoilage. British Poultry Science 18:661-670.

Mead, G.C. and Impey, C.S. (1984). Application of the competitive exclusion principle for preventing Salmonella colonisation in the newly hatched chick. Journal of Food Science 35:632-639.

Mendelson, H.A., Muller, V.J. and Treacy, G.B. (1984). Twisted states of Bacillus subtilis macrofibres reflect structural states of the cell. Proceedings of the National Academy of Science USA 81(1):3562-3566.

Moats, W.A. (1978). Egg washing: A review. Journal of Food Protection 41(11):919-925.

Mochizuki, A. and Matsumiya, M. (1983). Lysozyme activity in shell fishes. Bulletin of the Japanese Society Scientific Fisheries 49(1):131-136.

Morgan, F.J. and Arnheim, N. (1974). Preliminary biochemical studies on the lysozymes of the black swan. In Lysoyme Eds E.F. Osserman, R.E. Canfield and S. Beychok, S, pp 81-88. New York: Academic Press.

Morgan-Jones, S.C. (1982). The presence of salmonellae during the rearing of turkeys. British Poultry Science 23:481-486.

Mossel, D.A.A., Eelderink Ineke, de vor H. and Keizer, E.D. (1976). Use of agar immersion, plating and contact (AIPC) slides for the bacteriological monitoring of foods, meals and the food environment.

Laboratory Practice 25:393-395.

Nestor, K.E. and Bacon, W.L. (1981). Pimpling of egg shells and hatchability in several strains of turkeys. Poultry Science 60(8):1939-1943.

Norris, L.C. and Bauernfiend, J.C. (1940). effect of dietary riboflavin upon quantity stored in eggs and rate of exchange. Food Research 5:521-532.

Nurmi, E. and Rantala, M. (1973). New aspects of Salmonella infection in broiler production. Nature 241:210-211.

Oegema, T.R. and Jourdian, G.W. (1974a). The physical and chemical properties of a chicken egg white glycoprotein purified by non-denaturing methodology. Archives of Biochemistry and Biophysics 160:26-39.

Oegema, T.R. and Jourdian, G.W. (1974b). Metabolism of ovomucoid by the developing chick embryo. Journal of Experimental Zoology 189:147-161.

Ogden, A.L., Morton, J.R., Gilmour, D.G. and McDermid, E.M. (1962). Inherited variants in the transferrins and conalbumens of the chicken. Nature (Lond) 195:1026-1028.

Ohno, N., Yadomae, T. and Hyazaki, T. (1983). Resistant factors in Streptococcus pneumoniae cell wall to lysozyme digestion. Yakugaku Zasshi 103(11):1185-1189.

Orel, V. (1959). The pseudomonas spoilage of eggs laid by individual hens. Poultry Science 38:3-12.

Osbourne, T.E. and Campbell, C.F. (1900). The protein constituents of egg white. Journal of the American Chemical Society 22:422-450.

Ostlund, K. (1971). Bacteriology of washed and unwashed eggs. Acta veterinaria scandinavica 12:467-488.

Ostrowski, W., Skarzynski, B. and Zak, B. (1962). Isolation and the properties of flavoprotein from the egg yolk. Biochimica et biophysica acta 59:517-519.

Ostrowski, W., Zak, B. and Krawczyk, A. (1968). Riboflavin, flavoprotein from egg yolk - analytical and biophysical data. Acta biochimica polonica 15:243-260.

Paganelli, C.V., Ackerman, R.A. and Rahn, H. (1978). The Avian Egg : in vivo conductances of oxygen, carbon dioxide and water vapour in late development. In Respiratory Function in Birds, Adult and embryonic, Ed. J. Piiper, Berlin: Springer-Verlag.

Pai, C.H. and Lichstein, H.C. (1964). Observations on the use of

avidin in bacteriological media. Proceedings for the Society of Experimental Biology and Medicine 116:197-200.

Parascandole, C. (1893). Sur valore dell'albume d'uovo quale terreno di coltura dei microorganismi. Centrablatt fur Bakteriologie 14:219.

Parkinson, T.L. (1966). The chemical composition of eggs. Journal of the Science of Food and Agriculture 17:101-111.

Parsons, A.H. (1982). Structure of the eggshell. Poultry Science 61:2013-2021.

Paton, A.M. and Ayres, J.C. (1964). Use of fluorescent brightener for tracing the passage of Salmonella in eggs. Nature (Lond) 204:803-804.

Payne, J., Gooch, J.E.T. and Barnes, E.M. (1979). Heat resistant bacteria in pasteurised whole egg. Journal of Applied Bacteriology 46:601-613.

Pilet, P.E. and Bernasconi, P. (1984). Rubus hispidus callus cultured in vitro and endogenous lysozyme: Growth processes. Plant Science Letters 35(2):147-151.

Pivnick, H. and Nurmi, E. (1982). The Nurmi concept and its role in the control of salmonellae in poultry. In Developments in Food Microbiology-1 Ed. R. Davies, pp 41-70. London: Applied Science Publishers.

Pollack, J.R. and Neilands, J.B. (1970). Enterobactin - an iron transport compound from Salmonella typhimurium. Biochemical and Biophysical Research Communications 38:989-992.

Poole, S., West, S.I. and Walters, C.L. (1984). Protein-protein interactions: Their importance in the foaming of heterogenous protein enzymes. Journal of the Science of Food and Agriculture 35(6):701-711.

Powrie, W.D. (1977). Chemistry of eggs and egg products. In Egg Science and Technology Ed. W.J. Stadelman and O.J. Cotterill, pp 65-91. Connecticut: AVI Publishing.

Prager, E.M. and Wilson, A.C. (1974). Widespread distribution of lysozyme g in egg white of birds. Journal of Biological Chemistry 249:7295-7297.

Rahn, H., Paganelli, C.V. and Ar, A. (1974). The avian egg: Air-cell gas tension, metabolism and incubation time. Respiration Physiology 22:297-309.

Rahn, H., Ar, A. and Paganelli, C.V. (1979). How bird eggs breathe. Scientific American 240:46-55.

Rahn, H., Christensen, V.L. and Edens, F.W. (1981a). Changes in shell conductance, pores and physical dimensions of egg and shell during the

first breeding cycle of turkey hens. Poultry Science 60:2536-2541.

Rahn, H., Ledoux, T., Paganelli, C.V. and Smith, A.H. (1981b). Changes in the eggshell conductance and pore area after transfer of hens eggs from an altitude of 3800 to 1200m. Physiologist 24(4):135.

Rashid, A.M. (1981). The location of the structural difference between ovotransferrin types A and B in hens. Animal Blood Groups Biochemistry and Genetics 12(4):241-248.

Rettger, L.F. and Sperry, J.A. (1912). The antiseptic and bactericidal properties of egg white. Journal of Medical Research 26:55-64.

Reynolds, E.S. (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. Journal of Cell Biology 17:208-209.

Rhodes, M.B., Azari, P.R. and Feeney, R.E. (1958). Analysis, fractionation and purification of egg white protein with cellulose-cation exchanger. Journal of Biological Chemistry 230:399-409.

Rhodes, M.B., Bennett, N. and Feeney, R.E. (1959). The flavoprotein-apoprotein system of egg white. Journal of Biological Chemistry 234:2054-2060.

Rhodes, M.B., Bennett, N. and Feeney, R.E. (1960). The trypsin and chymotrypsin inhibitors from avian egg white. Journal of Biological Chemistry 235:1686-1693.

Rinehart, F.H. and Abul-Haj, G. (1951). An improved method for histologic demonstration of acid mucopolysaccharides in tissues. Archives of Pathology 52:189.

Rizzo, A. (1899). Sul numero e sulla distributione jei pori nel gucio dell'ovo di gallina. Richerchie del Anatomia las Roma 7:171-199.

Roberts, D. (1982). Factors contributing to outbreaks of food poisoning in England and Wales 1970-1979. Journal of Food Hygiene 89(3):491-498.

Robinson, D.S. and Monsey, J.B. (1971). Studies on the composition of egg white ovomucin. Biochemical Journal 121:537-547.

Robinson, D.S. and Monsey, J.B. (1972a). Changes in the composition of ovomucin during liquefaction of thick egg white. Journal of the Science of Food and Agriculture 23:29-38.

Robinson, D.S. and Monsey, J.B. (1972a). Changes in the composition of ovomucin during liquefaction of thick egg white. The effect of ionic strength and magnesium. Journal of the Science of Food and Agriculture 23:893-904.

Robinson, D.S. and Monsey, J.B. (1975). The composition and proposed subunit structure of egg white B-ovomucin. Biochemical Journal 147:55-62.

Roepke, R.R. and Bushnell, L.D. (1936). A serological comparison of the phosphoprotein of the serum of the laying hen and the vitelline of the egg yolk. Journal of Immunology 30:109-113.

Rogers, H.J. (1973). Iron-binding catechols and virulence in Escherichia coli. Infection and Immunity 7:445-456.

Rogers, H.J. and Perkins, H.R. (1968). Cell Walls and Membranes. London: E. and F.N. Spon Ltd.

Romankewitsch, N.A. (1934). Das Kutikulum des Huhnereies. Zeitschrift fur Zellforschung und mikroskopische Anatomie 21:110-118.

Romanoff, A.L. (1931). Why some eggs do not hatch. Cornell University Bulletin no 25.

Romanoff, A.L. (1943). Study of various factors effecting permeability of birds' eggshells. Food Research 8:212-223.

Romanoff, A.L. and Romanoff, A.J. (1949). The Avian Egg. New York: John Wiley.

Romanoff, A.L. and Romanoff, A.J. (1967). Biochemistry of the Avian

Embryo: A Quantitative Analysis of Prenatal Development. New York: John Wiley.

Rupley, J. (1966). The binding and cleavage by lysozyme of N-acetylglucosamine oligosaccharides. Proceedings of the Royal Society (Lond), series B 167:416-428.

Ryan, C.A., Clark, J.J. and Tomimatsu, Y. (1965). Chicken chymotrypsin and turkey chymotrypsin. (II) Physical and enzymatical properties. Archives of Biochemistry and Biophysics 110:175-183.

Salton, M.R.J. and Ghuysen, J.M. (1960). Acetylhexosamine compounds enzymatically released from Micrococcus lysodeikticus cell walls. III. The structure of di- and tetra-saccharides released from cell walls by lysozyme and streptomyces. Biochimica et biophysica acta 45:355-363.

Savage, W.G. and White, P.B. (1925). An investigation of the Salmonella group, with special reference to food poisoning. Medical Research Council Britain Special Report Series 91:1-157.

Schade, A.L. and Caroline, L. (1944). Raw hen egg white and the role of iron in growth inhibition of Shigella dysenteriae, Staphylococcus aureus, Escherichia coli and Saccharomyces cerivisiae. Science (New York) 100:14-15.

Schjeide, O.A., Wilkens, M., McCandless, R.G., Munn, R., Peterson, M.

and Carlson, E. (1963). Liver synthesis, plasma transport and structural alterations accompanying passage of yolk proteins. American Zoologist 3:167-184.

Schleifer, J.H. (1985). A review of the efficacy and mechanism of competitive exclusion for the control of salmonella in poultry. Worlds Poultry Science Journal 41(1):72-83.

Schmidt, W.J. (1958). Uber den Aufbau der Schale des Vogeleies nebst Bemerkungen uber Kalkige Eischalen anderer Tiere. Naturwissenschafliche Abteilung 28:82-108.

Schmidt, W.J. (1966). Coalescence of the calcite spherites during the formation of the avian eggshell. Zeitschrift fur Morphologie und Okologie der Tiere 52(2):252-258.

Seviour, E.M. and Board, R.G. (1972). The behaviour of mixed bacterial infections in the shell membranes of the hen's egg. British Poultry Science 13:33-43.

Sharp, P.F. and Powell, C.K. (1931). Increase in pH of white and yolk of hen's eggs. Journal of Industrial and Engineering Chemistry 23:196.

Sharp, P.F. and Whitaker, G.F. (1927). The relation of the hydrogen ion concentration of egg white to its germicidal action. Journal of Bacteriology 14:17-46.

Shaw, S. and Keddie, R.M. (1983). A numerical taxonomic study of the genus Kurthia with a revised description of Kurthia zopfii and a description of Kurthia gibsonii sp. nov. Systematic and Applied Microbiology 4:253-276.

Sibley, C.G. (1960). The electrophoretic patterns of avian egg white proteins as taxonomic characters. Condor 59:166-191.

Sibley, C.G. (1970). The electrphoretic patterns of the egg white proteins of passerine birds. Bulletin no 32 Peabody Museum of Natural History, Yale University.

Sibley, C.G. and Ahlquist, J.E. (1972). A comparative study of the egg white proteins of non-passerine birds. Bulletin no 39 Peabody Museum of Natural History, Yale University.

Silliker, J.H. (1982). The Salmonella problem: Current status and future direction. Journal of Food Protection 45(7):661-666.

Silyn-Roberts, H. (1983). Interior openings of functional pores in the avian eggshell: identification with the Scanning Electron Microscope. British Poultry Science 24:497-499.

Simkiss, K. (1968). The structure and formation of the shell and shell membranes. In Egg Quality: A Study of the Hens Egg, Ed. T.C. Carter, Edinburgh: Oliver and Boyd.

Simons, P.C.M. (1971). Ultrastructure of the hen eggshell and its physiological interpertation. Communication no 175 Central Institute Poultry Research Beekbergen, The Netherlands.

Simons, P.C.M. and Wiertz, G. (1963). Notes on the structure of membranes and shell in the hen's egg. An electron microscopical study. Zeitschrift Zellforschung mikroskopische Anatomie 59:555-567.

Simons, P.C.M. and Wiertz, G. (1970). Notes on the structure of shell and membrane of the hen's egg. A study with the Scanning Electron Microscope. Institute Pluimveeteelt Spelderholt Meded 161:31-49.

Skalinsky, E.I. (1966). The ultrastructure of the shell and the shell-membrane of chicken eggs. 6th International Congress for Electron Microscopy, Kyoto.

Smeltzer, T.I., Orange, K., Peel, B. and Runge, G. (1979). Bacterial penetration in floor and nest box eggs from meat and layer birds. Australian Veterinary Journal 55:592-593.

Smith-Gill, S.J., Mainhart, C.R., Lavoie, T.B., Potter, M. and Rudikoff, S. (1984). Vl-Vh expression by monoclonal antibodies recognising avian lysozyme. Journal of Immunology 132(2):963-967.

Sparks, N.H.C. and Board, R.G. (1984). Cuticle, shell porosity and water uptake through hen's eggshells. British Poultry Science

25:267-276.

Spiegel, A.M. (1966). In Statistical Methods in Biology Ed. N.T.J. Bailey, London: Hodder and Stoughton.

Spiro, T.G. and Saltman, P. (1969). Polynuclear complexes of iron and their biogical implications. Structure and Bonding (Berlin) 6:116-156.

Starcher, B.C. and King, G.S. (1980). The presence of desmosine and isodesmosine in eggshell membrane protein. Connective Tissue Research 8:53-55.

Stephens, F.C. and Feeney, R.E. (1963). Chemical Modification of avian ovomucoids. Biochemistry 2:1346-1352.

Stevens, A.J. (1971). Economic loss from poultry disease in Great Britain. In Poultry Disease and World Economy Eds. R.F. Gordon and B.M. Freeman, pp 25-36. Edinburgh: British Poultry Science.

Stewart, G.F. (1935). The structure of the hen's eggshell. Poultry Science 14:24-32.

Stokes, J.L. and Osbourne, W.W. (1956). Effect of the egg shell membrane on bacteria. Food Research 21:264-269.

Stokes, J.L., Osbourne, W.W. and Bayne, H.G. (1956). Penetration and growth of Salmonella in shell eggs. Food Research 21:510-518.

Stuart, J.C. (1984). The introduction and occurrence of Salmonella on production farms. Turkeys 29(5):25-27, 32.

Stuart, L.S. and McNally, E.H. (1943). Bacteriological studies on the egg shell. U.S. Egg Poultry Magazine 49:28-47.

Talbot, C.J. and Tyler, C. (1974). A study of the progressive deposition of shell in the shell gland of the domestic hen. British Poultry Science 15:217-224.

Tan, A.T. and Woodworth, R.C. (1969). Ultra-violet difference spectral studies of conalbumen complexes with transition metal ions. Biochemistry 8:3711-3716.

Todd, E. (1980). Poultry associated food-borne disease - its occurrence, cost, sources and prevention. Journal of Food Protection 43:129-139.

Tomimatsu, Y., Clary, J.J. and Bartulovich, J.S. (1966). Physical characteristics of ovoinhibitor, a trypsin and chymotrypsin inhibitor from chicken egg white. Archives of Biochemistry and Biophysics 115:536-544.

Tranter, H.S. (1982). The antimicrobial defence system of avian egg

albumen. PhD Thesis, University of Bath, England.

Tranter, H.S. and Board, R.G. (1982a). The antimicrobial defence of avian eggs: Biological perspective and chemical basis. Journal of Applied Biochemistry 4:295-338.

Tranter, H.S. and Board, R.G. (1982b). The inhibition of vegetative cell outgrowth and division from spores of Bacillus cereus T by hen egg albumen. Journal of Applied Bacteriology 52:67-73.

Tranter, H.S., Sparks, N.H.C. and Board, R.G. (1983). Changes in the structure of of the limiting membrane and in oxygen permeability of the chicken egg integument during incubation. British Poultry Science 24:537-547.

Tullett, S.G. (1975). Regulation of avian eggshell porosity. Journal of Zoology (Lond) 177:339-348.

Tullett, S.G. (1976). The avian eggshell - a mediating boundary. PhD Thesis, University of Bath, England.

Tullett, S.G. and Board, R.G. (1976). Oxygen flux across the integument of the avian egg during incubation. British Poultry Science 17:441-450.

Tullett, S.G., Lutz, P.L. and Board, R.G. (1975). The fine structure of the pores in the shell of the hen's egg. British Poultry Science

Tranter, H.S. and Board, R.G. (1984). The influence of incubation temperature and pH on the antimicrobial properties of hen egg albumen. Journal of Applied Bacteriology 56(1):52-62.

16:93-95.

Tullett, S.G. and Smith, S. (1976). A note on changes in egg shell porosity with flock age and season during the first breeding cycle of domestic ducks. British Poultry Science 24:501-509.

Tung, M.A., Garland, M.R. and Gill, P.K. (1979). A Scanning Electron Microscope study of bacterial invasion in hen's egg shell. Canadian Institute of Food Science and Technology Journal 12(1):16-22.

Turro, R. (1902). Zur Bakterienverdung. Zentralblatt fur Bakteriologie, Parasitenkunde, Infecktionskrankheiten und Hygiene (Abteilung I). 32:105-109.

Tyler, C. (1945). The porosity of egg shells, and the influence of different levels of dietary calcium upon porosity. Journal of Agricultural Science 35:168-176.

Tyler, C. (1953). Studies on egg shells. II. A method of marking and counting pores. Journal of the Science of Food and Agriculture 4:266-273.

Tyler, C. (1964). Wilhelm von Nathusius on Avian Eggshells. The University, Reading, England.

Tyler, C. (1964b). A study of the shells of the Anatidae. Proceedings of the Zoological Society (Lond) 149:547-583.

Tyler, C. (1965). A study of the eggshells of the Sphenisciformes. Journal of Zoological Research 147:1-19.

Tyler, C. (1966). A study of the eggshells of the Falconiformes. Journal of Zoological Research 150:413-425.

Tyler, C. (1969a). The distribution of pores in the eggshells of the domestic fowl: a further study. British Poultry Science 10:375-380.

Tyler, C. (1969b). A study of the eggshells of the Gaviiformes, Procellariiformes, Podicipitiformes and Pelecaniformes. Journal of Zoology (Lond) 158:395-412.

Tyler, C. and Fowler, S. (1978). The distribution of organic cores, cones, cone junctions and pores in the egg shells of wild birds. Journal of Zoology (Lond) 186:1-14.

Tyler, C. and Simkiss, K. (1959). A study of the eggshells of ratite birds. Proceeding of the Zoological Society (Lond) 133:201-243.

Vadehra, D.V., Baker, R.C. and Naylor, H.B. (1970). Role of cuticle in spoilage of chicken eggs. Journal of Food Science 35:5-6.

Villee, C.A., Walker, W.F. and Barnes, R.D. (1978). In General Zoology 5th Edit W.B., p 308. Philidelphia: Saunders and Co.

Völker, O. (1940). Die Verbreitung von Protoporphyrin in Vogeleischalen. Journal fur Ornithologie (Lpz) 88:604-612.

Walden, C.C., Allen, I.V.F. and Trussel, P.C. (1956). The role of the eggshell in restraining the entry of microorganisms. Poultry Science 35:1190-1196.

Wangensteen, O.D. and Rahn, H. (1970/1971). Respiratory gas exchange by the avian embryo. Respiration Physiology 11:31-45.

Wangensteen, O.D., Wilson, D. and Rahn, H. (1970/1971). Diffusion of gases across the shell of the hens egg. Respiration Physiology 11:16-30.

Waring, W.S. and Werkman, C.H. (1942). Growth of bacteria in an iron-free medium. Archives of Biochemistry 1:303-310.

Warner, R.C. and Weber, I. (1953). The metal combining properties of conalbumen. Journal of Biological Chemistry 75:5094-5101.

Wedral, E.M. (1971). Mechanism of bacterial penetration into the eggs of Gallus gallus. PhD Thesis, Cornell University, USA.

Wedral, E.M., Vadehra, D.V. and Baker, R.C. (1974). Chemical composition of the cuticle and the inner and outer shell membranes from eggs of Gallus gallus. Comparative Biochemistry and Physiology 47B:631-640.

Wei, R.D. and Wright, L.D. (1964). Heat stability of avidin and avidin-biotin complex and influence of ionic strength on affinity of avidin for biotin. Proceedings of the Society for Experimental Biology and Medicine 117:341-344.

Weibull, C. (1953). The isolation of protoplasts from Bacillus megaterium by controlled treatment with lysozyme. Journal of Bacteriology 66:688-695.

Weston, W.A.R.D. and Halnan, E.T. (1927). "Black spot" of eggs. Poultry Science 6:251-258.

White, H.B., Dennison, B.A., Fera, M.A.D., Whitney, C.J., McGuire, J.C., Meslar, H.W. and Sammeluitz, P.H. (1976). Biotin-binding protein from chicken egg yolk - assay and relationship to egg white avidin. Biochemical Journal 157:395-400.

Wilding, P. and Baxter-Jones, C. (1982). Controlling egg-borne pathogens. Poultry International June:12-22.

Williams, H.M. (1964). Role of the poultry industry. In Proceedings of the National Conference on Salmonellosis 1964, pp 158-160. U.S. Dept of Public Health.

Williams, J. (1962). A comparison of the transferrin and conalbumen in the domestic fowl. Biochemical Journal 83:355-364.

Williams, J., Elleman, T.C., Kingston, I.B., Wilkins, A.G. and Kuhn, K.A. (1982). The primary structure of hen ovotransferrin. European Journal of Buochemistry 122(2):297-304.

Williams, J.E. (1978). Recent literature on detection of salmonellae in poultry production flocks. A critical Review. Avian Pathology 7(1):1-14.

Williams, J.E. (1981a). Salmonellas in poultry feeds - a world wide review. Part I - II. Worlds Poultry Science Journal 37(1):6-20.

Williams, J.E. (1981b). Salmonellas in poultry feeds - a world wide review. Part III. Worlds Poultry Science Journal 37(2):97-105.

Williams, J.E. and Whittemore, A.D. (1967). A method for studying microbial penetration through the outer structures of the avian egg. Avian Diseases 11:467-490.

Williams, S.C. and Woodworth, R.C. (1973). The interaction of iron-conalbumen (anion) complexes with chick embryo red blood cells. Journal of Biological Chemistry 248:5848-5853.

Wing-Ming, K. and Azari, P. (1982). Structure and function of ovotransferrin: 2. Iron-transferring activity of iron-binding fragments of ovotransferrin with chicken embryo red cells. Journal of Biological Chemistry 257(3):1184-1188.

Wing-Ming, K., Azari, P. and Philips, J.L. (1982). Structure and function of ovotransferrin: 1. Production of iron-binding fragments from iron-ovotransferrin by the action of immobilised subtilisin: purification and characterisation of the fragments. Journal of Biological Chemistry 257(3):1177-1183.

Winter, W.P., Buss, E.G., Clagett, C.O. and Boucher, R.V. (1967a). The nature of the biochemical lesion in avian renal riboflavinuria - 1. Effect of genotype on renal riboflavin metabolism. Comparative Biochemistry and Physiology 22:889-896.

Winter, W.P., Buss, E.G., Clagett, C.O. and Boucher, R.V. (1967b). The nature of the biochemical lesion in avian renal riboflavinuria - 2. The inherited change of a riboflavin binding from blood and eggs. Comparative Biochemistry and Physiology 22:897-906.

Wolk, J., McNally, E.H. and Spicknall, N.H. (1950). The effect of temperatures on bacterial infection of shell eggs. Food Technology 4:316-318.

Wolken, J.J. (1951). Structure of hen's egg membranes. Anatomical Reviews 111:79-89.

Wooley, D.W. and Longsworth, L.G. (1942). Isolation of an antibiotic factor from egg white. Journal of Biological Chemistry 142:285-290.

Wurtz, R. (1890). De l'action bactericide du blanc d'oeuf. Semaine Medicale (Paris) 3:21.

Wyburn, G.M., Johnston, H.S., Draper, M.H. and Davidson, M.F. (1973). The ultrastructure of the egg forming region of the oviduct and the development of the shell of Gallus domesticus. Quarterly Journal of Experimental Physiology 58:143-151.

Yeh, Y., Iwai, S. and Feeney, R.E. (1979). Conformations of denatured and renatured ovotransferrin. Biochemistry 18(5):882-889.

Zagaevsky, J.S. and Lutikova, P.O. (1944). Sanitary measures in the egg breaking plant. U.S. Egg and Poultry Magazine 50:17-20, 43-46, 75-77, 88-90, 121-123.

Zinder, N.D. and Arndt, W.F. (1956). Production of protoplast of Escherichia coli by lysozyme treatment. Proceedings of the National Academy of Sciences U.S.A. 42:586-590.

Zipperle, G.F., Ezzell, J.W. and Doyle, R.J. (1984). Glucosamine substitution and muramidase susceptibility in Bacillus anthracis. Canadian Journal of Microbiology 30(5):553-559.

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CHANGES IN STRUCTURE OF THE LIMITING MEMBRANE AND IN OXYGEN PERMEABILITY OF THE CHICKEN EGG INTEGUMENT DURING INCUBATION

H. S. TRANTER, N. H. C. SPARKS AND R. G. BOARD

School of Biological Sciences, Bath University, Bath BA2 7AY, Avon, England

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1. Oxygen permeabilities (K_{O_2}) of the shell and shell membranes of fertile and infertile chicken eggs were measured at 37·5 °C and a relative humidity of 0·60 throughout 14 d incubation, with turning. The K_{O_2} of the shell and membranes of infertile eggs was around $1{\cdot}0 \times 10^{-7}$ cm^3 O_2STP sec^{-1} cm^{-2} Torr^{-1} (1 Torr = 133·322 Pa) throughout incubation. With fertile eggs, from which there was a linear loss of water during incubation, the K_{O_2} of the shell and shell membranes was about $1{\cdot}0 \times 10^{-7}$ cm^3 O_2STP sec^{-1} cm^{-2} Torr^{-1} for the first four days of incubation. Thereafter the majority of shells and membranes had a K_{O_2} of about $1{\cdot}0 \times 10^{-6}$ cm^3 O_2STP sec^{-1} cm^{-2} Torr^{-1}.

2. A diminution of the Na^+ and K^+ content of the shell membranes of fertile eggs was not associated with changes in the dimensions of the glycoprotein mantle on the cores of the individual fibres of the membranes. There was, however, a progressive deterioration in the limiting membrane of fertile but not of infertile eggs.

3. It was concluded that changes in the O_2 resistance of the integument of fertile eggs were not a product of change in either of the shell membranes but of damage caused to the limiting membrane by the chorioallantois.

INTRODUCTION

Several studies have shown that a 10-fold increase in the oxygen permeability of the integument (the porous calcitic shell lined on its outer surface with cuticle and on its inner surface with fibrous membranes) occurs 22% of the way through incubation of the fertile eggs of several species of birds (Kutchai and Steen, 1971; Lomholt, 1976; Tullett and Board, 1976; Kayar *et al.*, 1981), providing that the integument does not dry out before or during determinations (Wangensteen *et al.*, 1970/1971). Indeed attempts to interpret this observation have invoked discussion about the role of water, either in or between the fibres of the shell membranes, on the diffusion of oxygen through the interstitial spaces. Thus with the latest study (Kayar *et al.*, 1981), the

hypothesis was advanced that the increased permeability of the inner membrane, which these authors found to account for 88% of the initial resistance of the integument to oxygen flux, was due to evaporation causing a layer of water in this membrane to be reduced in thickness from about 63 to 0·6 μm.

Before any hypothesis that attempts to explain changes in the oxygen permeability of shell membranes in terms of the latter's content of water is used to plan further experiments, it is pertinent to consider both the fine structure and chemical composition of the shell membranes. Although the literature quoted above conveys the impression that the avian shell is lined with only two membranes, Bellairs and Boyde (1969) found in studies of fine structure with the electron microscope that the inner surface of the main shell membrane is covered with electron dense material, which they called the limiting membrane. It is notable also that a narrow band of electron dense material separating the main shell membrane and the chorioallantois has been observed repeatedly (Leeson and Leeson, 1963; Skalinsky and Kondalenko, 1963; Froix *et al.*, 1977). Thus in practice there are good reasons for considering that there are three resistances, the inner and outer shell membranes and the limiting membrane, to the inward diffusion of oxygen that has passed through the pores in the calcitic shell. Moreover the diameters of the fibres of the first two membranes vary appreciably, those in the outer shell membrane being upwards of 3 to 6 μm and those of the inner being as little as 0·4 μm. Furthermore the fibres have a composite structure, a central core of a fibrous protein rich in desmosine and isodesmosine (Harris *et al.*, 1980) but resistant to elastase (Leach and Rucker, 1978; Starcher and King, 1980; Crombie *et al.*, 1981; Leach *et al.*, 1981) is surrounded by a glycoprotein mantle which probably contains glucose, galactose, mannose, xylulose, glucosamine, galactosamine and sialic acid (Baker and Balch, 1962; Cooke and Balch, 1970; Wedral *et al.*, 1974). The present study was undertaken with the objectives of studying the fine structure of the shell and limiting membranes of the incubating eggs of the domestic chicken and attempting to associate the diminution in the resistance of the egg's integument to oxygen flow with changes in fine structure.

MATERIALS AND METHODS

Eggs

Eggs of the domestic hen were obtained from local farm hens housed in batteries (infertile eggs) or from a local commercial hatchery (fertile eggs). The eggs were used immediately or incubated in a small incubator (Brinsea Products Ltd, West Brinsea Farm, Congresbury, Avon) maintained at 37·5 °C and 0·60 relative humidity (RH). The eggs were turned automatically every hour.

Glassware

All glassware was thoroughly cleaned by soaking in concentrated nitric acid overnight and washing in deionised, glass-distilled water.

Measurement of K_{O_2}

The oxygen flux across the shell and shell membranes of both fertile and infertile hen eggs was measured using the apparatus described by Kutchai and Steen (1971).

Eggs at various stages of incubation were cut around their shoulder and the narrow pole wiped clean of adhering albumen with a tissue. The shell was immediately placed in the apparatus and sealed around the shoulder with "Xantoprene" polymer (Bayer Ltd). The enclosed space beneath the shell was flushed with humidified N_2 until the O_2 concentration as measured by the electrode of an O_2 meter (Walden Precision Apparatus Ltd, Saffron Walden, Essex) was zero. The gas inlet and outlets were closed and the time taken to reach half the maximum O_2 concentration recorded on paper charts.

The surface area of the egg shells was determined by breaking them up into small pieces and passing these through an automatic surface area recorder (Lambda Instruments Corporation, Lincoln, Nebraska, USA). The results were expressed as described by Wangensteen *et al.* (1970/1971).

Ionic content of shell membranes

Eggs were cracked open and their contents of white and yolk removed. The shell membranes were washed ten times with deionised glass-distilled water, removed from the shell, and dried to a constant weight in a hot-air drying oven at 80 °C. The dried membranes were ground to a fine powder using a glass pestle and mortar and ashed in a muffle furnace overnight at 450 °C or until a whitish-grey ash remained. If difficulty was experienced in obtaining a carbon-free ash, the ash was cooled, moistened with deionised water, evaporated to dryness at 80 °C and reheated to 450 °C. When all the organic matter was destroyed the ash was cooled and a solution of the ash prepared as for plant material (Agricultural Development and Advisory Service, 1973).

Sodium and potassium were analysed using the flame photometer (Coning Ltd, Halstead, Essex) and calcium and magnesium analysed by atomic absorption using an atomic absorption spectrophotometer (SP1600, Pye-Unicam Ltd). In both cases standard solutions of the elements (Fisons Ltd) were used as described by the Agricultural Development and Advisory Service (1973).

Electron microscopy

Samples of shell membranes from the broad pole of eggs for scanning electron microscopy were fixed in 0·19 M gluteraldehyde in cacodylate buffer (pH 7·0) for 60 min at room temperature followed by dehydration in a series (8·60 to 17·21 M) acetone. The samples were dried to critical point using a critical point dryer (Polaron Equipment Ltd, Watford, London) and mounted on aluminium stubs. The specimens were coated with a thin layer of gold/palladium alloy under vacuum and examined using a scanning electron microscope (35C, Jeol (UK) Ltd, Colindale, London) at an accelerating voltage of 25 kV.

Samples of shell with membranes attached were prepared by freezing large sections of shell in liquid N_2 and then fracturing them into small pieces which were subsequently freeze dried (Edwards, Crawley, Sussex) and mounted on aluminium plates. The samples were then coated as described above and examined using the scanning electron microscope.

Samples of shell membrane for transmission electron microscopy were fixed and stained in 0·19 M gluteraldehyde containing 1 g ruthenium red/l. After dehydration as described above the membranes were embedded in Taab EM resin (Taab Labora-

tories Equipment Ltd, Reading, Berkshire) and ultrathin sections, cut with a glass knife, collected onto uncoated copper grids. Sections were stained with a saturated solution of uranyl acetate in ethanol/water (700 ml/l) and Reynolds lead citrate for 15 min each and examined using a transmission electron microscope (Jeol 100C) at an accelerating voltage of 25 kV.

RESULTS

Oxygen permeability

Oxygen permeability (K_{O_2}) through the integuments of 33 infertile eggs was about $1{\cdot}0 \times 10^{-7}$ cm^3 O_2STP sec^{-1} cm^{-2} Torr^{-1} throughout the 14 d of incubation at 37·5 °C and 0·60 RH (Fig. 1). The 24 fertile eggs examined up to the 4th day of incubation had K_{O_2} values similar to those of the infertile ones. After day 4, however, the resistance of the integuments of fertile eggs to oxygen diffusion diminished by about 10-fold and all the eggs examined gave K_{O_2} values of about $1{\cdot}0 \times 10^{-6}$ cm^3 O_2STD sec^{-1} cm^{-2} Torr^{-1} by the 10th day of incubation. These results, which are in

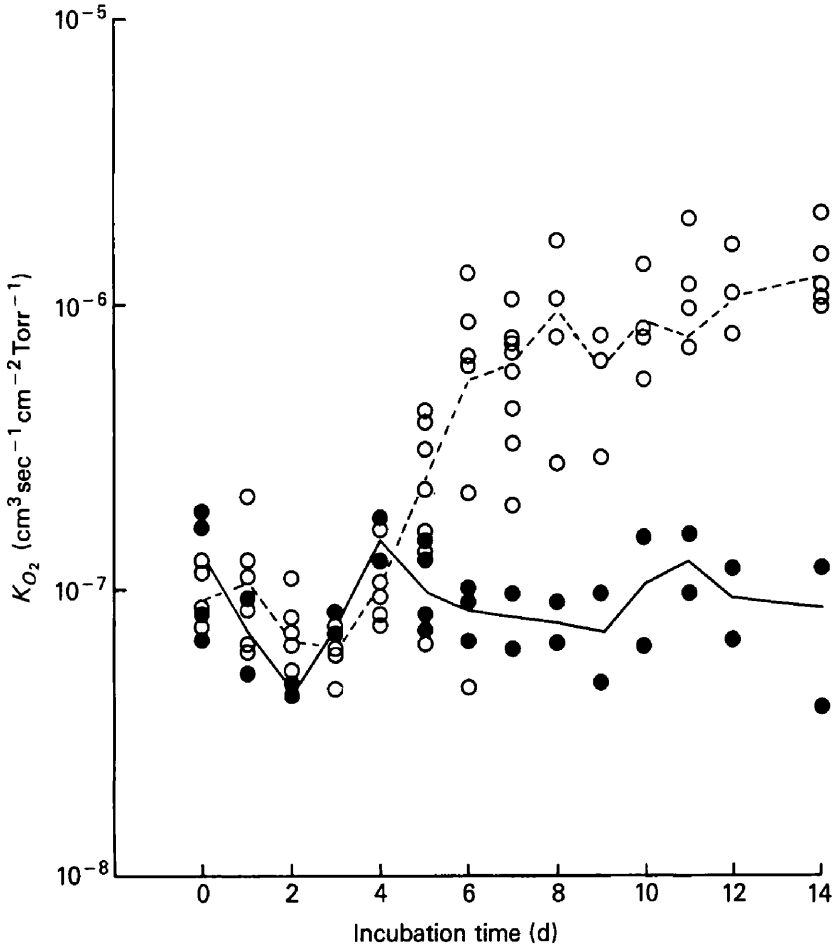

FIG. 1.—The rate of oxygen transfer across the shell and shell membranes of the infertile (closed circles and solid line) and fertile (open circles and broken line) eggs of the domestic hen at different times during incubation. Each symbol is the result obtained with one egg incubated (37·5 °C) with turning for the period shown.

accord with those of Kutchai and Steen (1971); Lomholt (1976); Tullett and Board (1976); Kayar *et al.* (1981), show clearly that some change(s) occur in the integument of fertile hens' eggs during days 4 to 6 of incubation such that the potential for the inward flux of oxygen is increased. These changes were not reflected in the rate at which fertile eggs lost water by evaporation (Fig. 2).

Structure of shell membranes

It was evident in radial sections of the membranes taken from the shells of freshly laid eggs, both fertile and infertile, that there were three distinct layers (Plate-Fig. 1A), the outer two (the shell membranes *sensu strictu*) being distinguished one from the other by the diameter of their fibres, the larger ones being most numerous in the outer shell membrane. The limiting membrane, a narrow band of electron dense material, was a

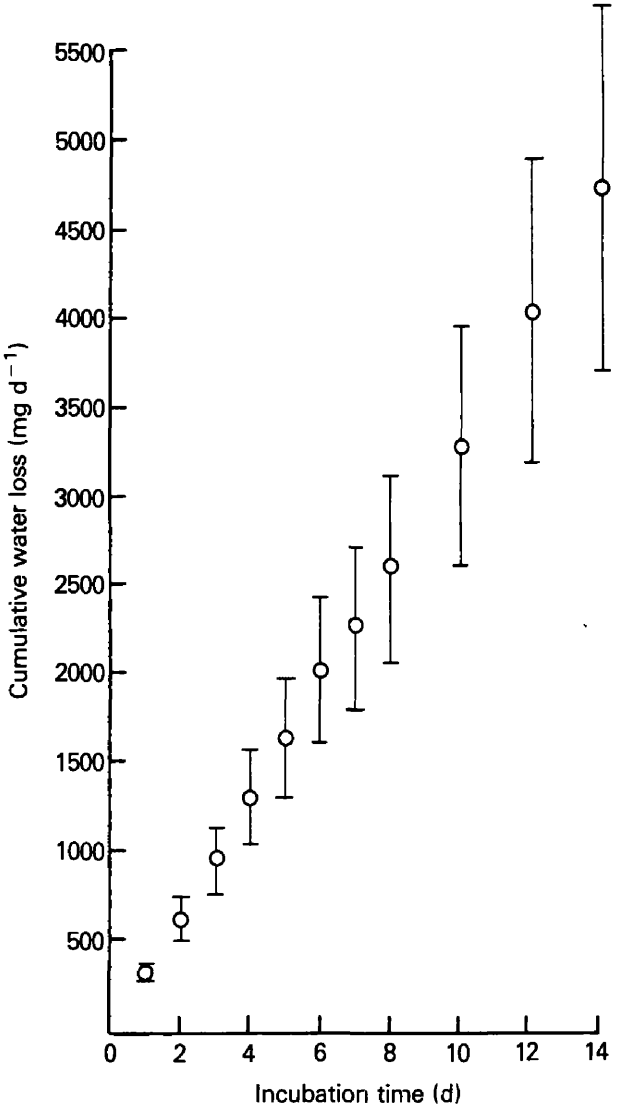

FIG. 2.—The weight loss from twelve fertile eggs of the domestic hen during incubation (37·5 °C) with turning for 14 d. Open circles, means; bars, range.

notable feature also (Plate-Fig. 1A). The core and mantle of individual fibres of the inner and outer shell membranes were evident with high powered magnification of ultra-thin sections stained with ruthenium red (Plate-Fig. 1B). Indeed, a limited survey of the shell membranes of the eggs of several species of water fowl (Sparks and Board, unpublished observations) and other bird species (Dr L. J. Alberto, personal communication) indicates that composite fibres may be a common feature in avian eggs. It was noted during studies with a scanning electron microscope that differential fracture of the shell membrane fibres revealed several instances of two or more protein fibres contained within a common mantle (Plate-Fig. 1B). Changes in the morphology of the shell membranes, especially in respect of the thickness of the mantle or the fibre, were not seen in incubated fertile or infertile eggs.

The limiting membrane appeared as an electron dense layer on the inner surface of the inner shell membranes examined by scanning electron microscopy (Plate-Fig. 2A). Its appearance in infertile eggs did not change during 14 d incubation with egg turning at 37·5 °C and 0·60 RH. In marked contrast there was a progressive change in the microstructure of the limiting membrane of fertile eggs in which embryos developed (Plate-Fig. 2A, B and C). It needs to be stressed that all material studied with electron optics was taken from the broad end of the shell, the pointed end being used for determination of K_{O_2}. The first demonstrable changes, cracks in the limiting membrane (Plate-Fig. 2B), were found in fertile eggs incubated for 4 d. As incubation proceeded, the cracks became wider and, by the 18th day of incubation, only patches of the limiting membrane on the underlying fibrous inner shell membrane were evident (Plate-Fig. 2C).

Cation content of the shell membranes

Of the cations studied, the concentrations of Na^+ and K^+ in the shell membranes of fertile eggs diminished significantly during the first 3 d of incubation, but that of Na^+ increased thereafter, and that of Mg^{2+} decreased between days 3 and 9. In contrast (Fig. 3), the concentrations of these cations in the membranes of infertile eggs decreased (Na^+) or increased (K^+, Mg^{2+}) slightly during the first 18 d of incubation. The marked increase in the concentrations of K^+ and Na^+ by day 21 was attributed to the yolk making contact with the shell membranes of infertile eggs. The increase in the concentrations of Ca^{2+} and Mg^{2+} in the membranes of fertile eggs on day 21 probably reflects the carryover of tips of the cones from the inner surface of the shell. It is known (Board and Love, 1980) that the cones are relatively rich in Mg^{2+} and that their resistance to fracture is reduced as a consequence of the embryo absorbing Ca^{2+} from the shell (Bond, 1980).

DISCUSSION

The results presented favour the conclusion that the decrease in the resistance of the integument of fertile hens' eggs to O_2 diffusion, which occurs about 22% of the time into incubation (Kutchai and Steen, 1971; Lomholt, 1976; Tullett and Board, 1976; Kayar *et al.*, 1981), is caused, either wholly or in part, by changes in the limiting membrane. Before accepting evidence of change in the structure of such a delicate structure, we examined many limiting membranes from incubated fertile as well as

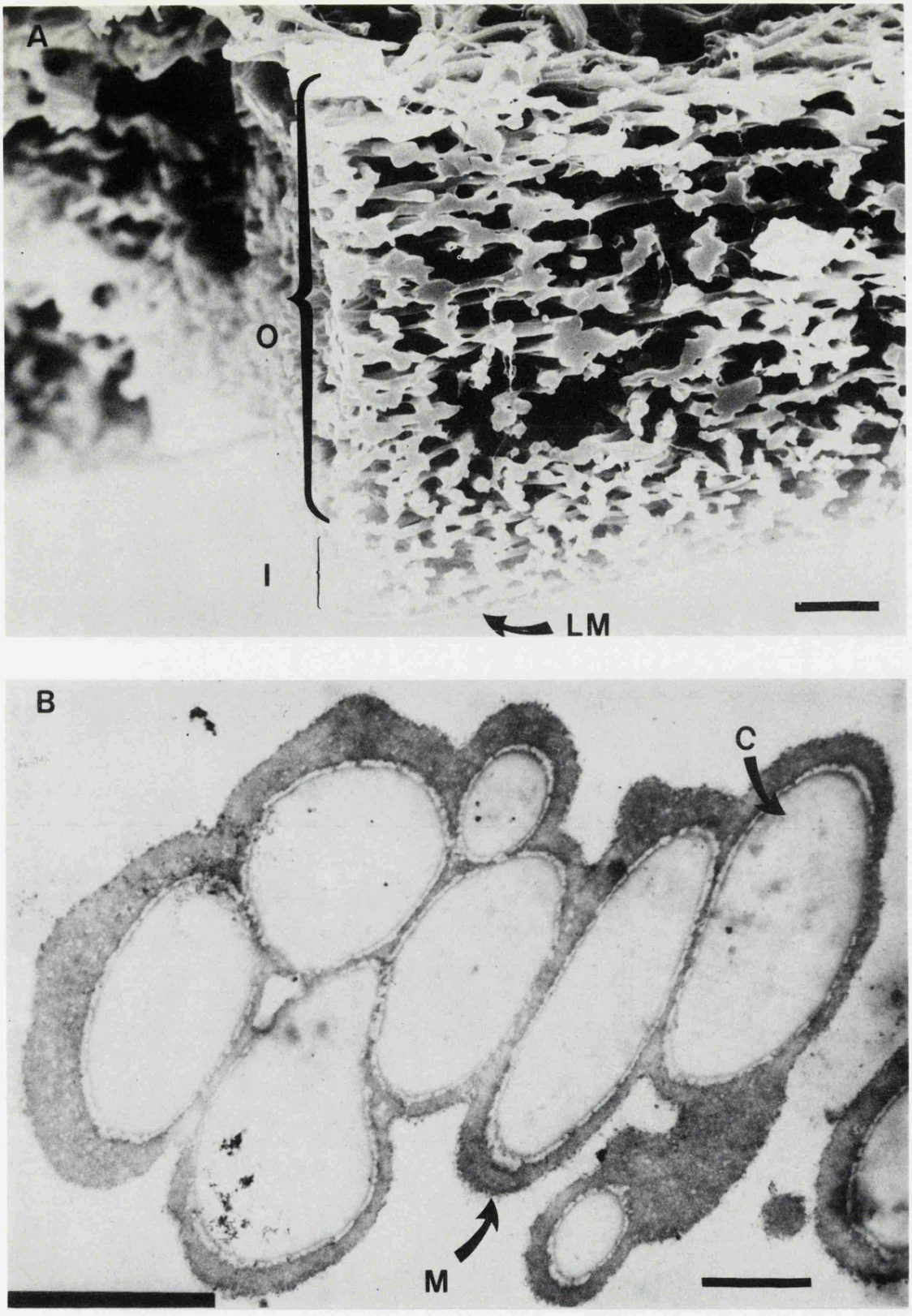

PLATE-FIG. 1.—Electron microscope examination of the shell membranes of the eggs of domestic hens. A. Scanning electron micrograph of a radial section of shell membranes prepared by freeze fracture and freeze drying; O, outer shell membrane (thick fibres), I, inner shell membrane (thin fibres), and LM, limiting membrane (electron dense material): bar marker, 10 μm. B. Transmission electron micrograph of a thin radial section (stained with ruthenium red) of the fibres in the shell membrane; C, cortex; M, mantle: bar marker, 1·0 μm.

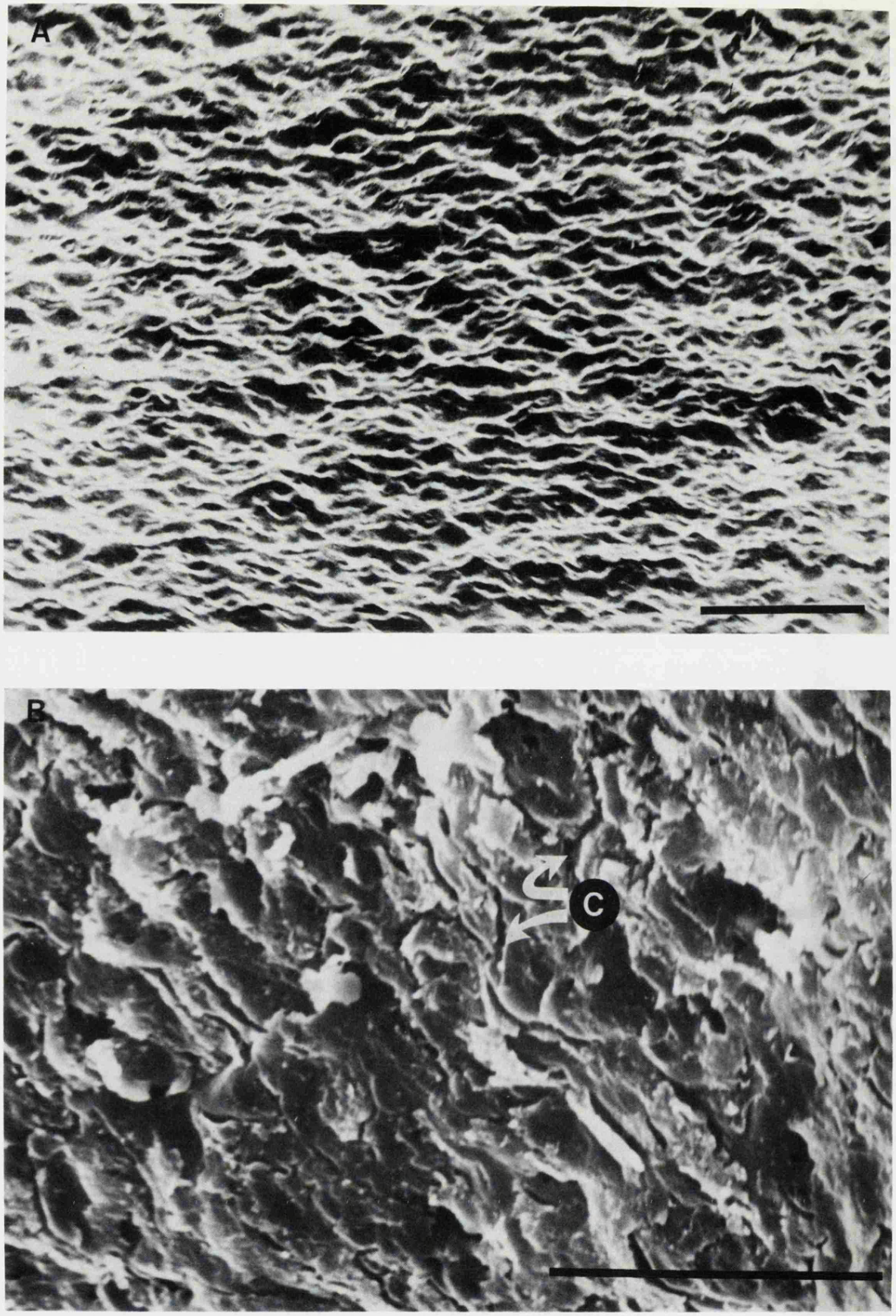

PLATE-FIG. 2.—Scanning electron micrograph of the limiting membrane of the eggs of domestic hens incubated (37·5 °C) with turning. A. The electron dense limiting membrane in infertile and fertile eggs incubated for up to 4 d appeared to be intact. B. and C. Cracks (C) and, subsequently, large gaps appeared in the limiting membrane (M), exposing the underlying inner membrane (F) of fertile hen's eggs in which there was a marked change (Fig. 1) of the K_{O_2} of the shell and shell membranes: bar marker, 10 μm.


F
M

lation between a decrease in a membrane's resistance to oxygen diffusion and the occurrence of cracks. Additional support for the contention that there is an increase in O_2 diffusion across half-shells of incubated eggs (see Methods) comes from the studies of Temple and Metcalfe(1970) and Kayar *et al.* (1981). The former reported a progressive decrease in the resistance of the shell, shell membranes and chlorioallantois to oxygen diffusion in studies with whole eggs. Kayar *et al.* (1981) obtained good agreement between the mean K_{O_2} calculated from analysis of the difference between the P_{O_2} of the atmosphere and the P_{O_2} of the air cell of fertile eggs incubated for 10 to 17 d and the K_{O_2} calculated from measurements with half-shells and an oxygen electrode (*i.e.* the same method as that used here). Thus two lines of evidence support the notion that changes in resistance to O_2 with incubation are the consequence of changes in the shell membranes occurring during incubation and not during the preparative/recording stages of the experiments discussed here and elsewhere (Kutchai and Steen, 1971; Lomholt, 1976; Tullett and Board, 1976).

Although we present evidence that changes in the limiting membrane cause a decrease in oxygen resistance, previous workers (Kutchai and Steen, 1971; Lomholt, 1976; Tullett and Board, 1976; Kayar *et al.*, 1981) have centred their discussions on the contribution of a water film to the membranes' resistance to oxygen flux. Thus Kayar *et al.* (1981) calculated that this film is 63 μm thick in eggs incubated for less than 4 d but only 0·6 μm in those incubated for 12 to 17 d and considered it plausible that this thinning occurred in the inner shell membrane alone. Not only are the discussions of a water film based on inference, they fail to take into account the microstructure of the shell membranes and the possible physicochemical contribution of the mantles on the individual membranes. As is evident in Plate-Fig. 1A, there is a progressive diminution in the inter-fibre distances and fibre diameters from the outside to the inside of the inner shell membrane. It would seem reasonable to assume from the studies of the behaviour of water in soils (Hillel, 1980) that if there was a progressive thinning of a water film in the membrane then the resistance to evaporative loss would increase, providing the mantles did not in some way modify the physical properties of the water connecting them. There was no evidence of such an increase in Fig. 2. Moreover if drying alone was responsible for the decrease in oxygen resistance noted in this study and those quoted above, why did it not occur in infertile eggs incubated alongside fertile ones (Fig. 1)?

The conclusion of Kayar *et al.* (1981) that the inner shell membrane accounts for 88% of the initial resistance of the egg's integument to oxygen diffusion would appear to be in need of amendment in the light of the results discussed above. In practice their estimate that it contributes only 12% of the total resistance in eggs incubated for more than 4 d is probably correct, the 66% of the resistance noted in freshly-laid fertile eggs and transiently in those incubated for 4 d, or less, being due to the limiting membrane. In summary, therefore, the impediments to oxygen diffusion across the cuticle, porous calcitic egg shell and the underlying membranes can be considered as resistances in series (Fig. 4). Of the six potential resistances shown in this diagram, that due to the limiting membrane (number 6) appears to be temporal only in incubating eggs.

As the evidence presented in this paper has identified the limiting membrane as an important resistance to oxygen diffusion, it is pertinent to consider mechanisms that diminish its resistance through propagating crack formation. In planning the present studies, attention was directed at the possibility of time-induced changes in the thick-

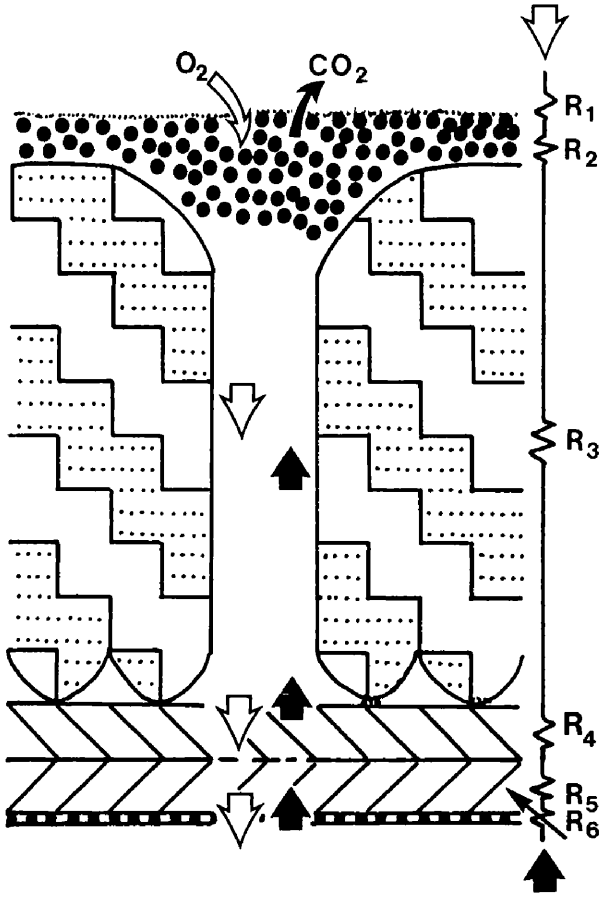

FIG. 4.—The diffusion pathway through the shell and shell membranes of the egg of the domestic hen viewed as resistances in series: R_1, boundary layer; R_2, cuticle; R_3, pore canal; R_4, outer shell membrane; R_5, inner shell membrane, and R_6, limiting membrane.

ness of the mantles of the fibres, it being assumed that a reduction in mantle thickness would enlarge the spaces between fibres. It is known, moreover, that the space filled by the glycoprotein cortex of the bacterial endospore (Gould and Pring, 1975) is determined by the nature of the cations involved in cross-linking. Thus the cortex occurs in the expanded form when K^+ and Na^+ are present but in the contracted form with Ca^{2+}. Although we demonstrated a diminution in the Na^+ and K^+ content (Fig. 3) of the shell membranes of incubated fertile but not infertile eggs, there was no corresponding change in the dimensions of the mantles. Moreover we are of the opinion that changes in the cation content of the mantles resulted from those in the concentration of these cations in underlying albumen. According to Tullett and Board (1976) the latter changes are associated with the transfer of water from the albumen during the formation of the sub-embryonic fluid and we speculate that cation loss from the shell membranes reflects transfer caused by a diffusion gradient being established between the shell membranes and the albumen. As there was no evidence of change in the size of the fibre mantles with incubation, the cracks in the limiting membrane cannot be attributed to mechanical stresses caused by changes in the support provided by the inner shell membrane.

We speculate that the cracks occurring in the limiting membrane are caused by mechanical stress due to egg turning. Future studies are needed to discriminate be-

tween stress due to the membrane being rubbed with albumen thickened as a consequence of water loss to the subembryonic fluid or the developing chorioallantois.

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REFERENCES

AGRICULTURAL DEVELOPMENT AND ADVISORY SERVICE (1973). *The Analysis of Agricultural Materials.* Ministry of Agriculture, Fisheries and Food, Technical Bulletin 27. London, Her Majesty's Stationery Office.

BAKER, J. R. AND BALCH, D. A. (1962). A study of the organic material of hen's egg shell. *Biochemical Journal*, **82**: 352–361.

BELLAIRS, R. AND BOYDE, A. (1969). Scanning electron microscopy of the shell membranes of the hens' egg. *Zeitschrift für Zellforschung und Mikroskopische Anatomie*, **96**: 237–249.

BOARD, R. G. AND LOVE, G. (1980). Magnesium distribution in avian eggshells. *Comparative Biochemistry Physiology*, **66A**: 667–672.

BOND, G. M. (1980). Avian eggshells and the hatching process. *Ph.D. Thesis, University of Bath.*

COOKE, A. S. AND BALCH, D. A. (1970). Studies of membrane, mammillary cores and cuticle of the hen egg shell. *British Poultry Science*, **11**: 345–352.

CROMBIE, G., SNIDER, R., FARIS, B. AND FRANZBLAU, C. (1981). Lysine-derived cross-links in the egg shell membrane. *Biochimica et Biophysica Acta*, **640**: 365–367.

FROIX, M. F., POCHAN, J., GOEDDE, A. AND HINMAN, D. L. (1977). A dynamic characterization via relaxation techniques of the shell membranes of the domesticated fowl (*Gallus/gallus* (L.)). *Polymer*, **18**: 1213–1218.

GOULD, G. W. AND PRING, G. J. (1975). Heat resistance of bacterial endospores and concept of an expanded osmoregulatory cortex. *Nature, London*, **258**: 402–405.

HARRIS, E. D., BLOUNT, J. E. AND LEACH, R. M. (1980). Localization of lysyl oxidase in hen oviduct: implications in eggshell membrane formation and composition. *Science*, **208**: 55–56.

HILLEL, D. (1980). *Fundamentals of Soil Physics.* London, Academic Press.

KAYAR, S. R., SNYDER, G. K., BIRCHARD, G. F. AND BLACK, C. P. (1981). Oxygen permeability of the shell and membranes of chicken eggs during development. *Respiration Physiology*, **46**: 209–221.

KUTCHAI, H. AND STEEN, J. B. (1971). Permeability of the shell and shell membranes of hens' egg during development. *Respiration Physiology*, **11**: 265–278.

LEACH, R. M. AND RUCKER, R. B. (1978). Studies on the major protein component of egg shell membranes. *Poultry Science*, **67**: 1151.

LEACH, R. M., RUCKER, R. B. AND VAN DYKE, G. P. (1981). Egg shell membrane protein: a non-elastin desmosine/isodesmosine-containing protein. *Archives of Biochemistry Biophysics*, **207**: 353–359.

LEESON, T. S. AND LEESON, C. R. (1963). The chorio-allantois of the chick. Light and electron microscopic observations at various times of incubation. *Journal of Anatomy*, **97**: 585–595.

LOMHOLT, J. P. (1976). The development of the oxygen permeability of the avian egg shell and its membranes during incubation. *Journal of Experimental Zoology*, **198**: 177–184.

SKALINSKY, E. I. AND KONDALENKO, V. F. (1963). Electron microscopic studies of the chick chorioallantois during embryogenesis. *Acta Morphologica*, **12**: 247–259.

STARCHER, B. C. AND KING, G. S. (1980). The presence of desmosine and isodesmosine in eggshell membrane protein. In: *International Reviews of Connective Tissue Research*, vol. 8, pp. 53–55. Edit. HALL, D. A. AND JACKSON, D. S. London, Academic Press.

TEMPLE, C. F. AND METCALFE, J. (1970). The effects of increased incubator oxygen tension on capillary development in the chick chorioallantois. *Respiration Physiology*, **9**: 216–233.

TULLETT, S. G. AND BOARD, R. G. (1976). Oxygen flux across the integument of the avian egg during incubation. *British Poultry Science*, **17**: 441–450.

WANGENSTEEN, O. D., WILSON, D. AND RAHN, H. (1970/71). Diffusion of gases across the shell of the hen's egg. *Respiration Physiology*, **11**: 16–30.

WEDRAL, E. M., VADEHRA, D. V. AND BAKER, R. C. (1974). Chemical composition of the cuticle, and the inner and outer shell membranes from eggs of *Gallus gallus*. *Comparative Biochemistry Physiology*, **47B**: 631–640.

British Poultry Science, **24**: 123–130. 1983

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A NOTE ON THE STRUCTURE AND IRON-BINDING PROPERTIES OF EGG-SHELL MEMBRANES

H. S. TRANTER, N. H. C. SPARKS AND R. G. BOARD

School of Biological Sciences, University of Bath, Bath BA2 7AY, Avon, England

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1. When a solution of ferric ammonium sulphate was added to shell membranes of the domestic fowl, iron infiltrated the mantle (cortex) surrounding the cores of the individual fibres of the membranes.

2. Contraction of warm eggs in ice-cold colloidal iron caused flooding of pore canals and contamination of the underlying shell membranes with this element.

3. Appreciable contamination of the inner shell membrane with iron persisted for 25 d in infertile eggs stored at 37·5 °C.

INTRODUCTION

The egg-shell membranes of the domestic fowl consist of three distinct layers. The major ones, the inner and outer membranes, are formed from a network of randomly orientated fibres having their long axes parallel to the surface of the egg shell (Bellairs and Boyde, 1969). The diameter of the fibres range from 0·4 to 3·6 μm, the smaller ones being more numerous in the inner membrane. A homogenous third layer of electron dense material, the limiting membrane of Bellairs and Boyde (1969), lines the inner surface of the inner shell membrane.

Although the general morphology of the egg-shell membranes and their possible role in the transfer of Ca^{2+} from the shell of the embryo via the chorioallantois have been studied extensively (Masshoff and Stolpmann, 1961; Simkiss, 1968; Tung and Richards, 1972; Narbaitz and Tellier, 1974), little is known about their chemical composition particularly with respect to fine structure. The fibres of the inner and outer shell membranes have a protein core (the medulla) surrounded by a mucopolysaccharide mantle or cortex. Early investigators (Moran and Hale, 1936) concluded that the membranes contained a fibrous protein, probably keratin. The demonstration that the shell membranes are rich in cysteine (Baker and Balch, 1962) would support such a view. Recently, however, histochemical and ultrastructural analyses (Hoffer, 1971; Wedrel *et al.*, 1974) have not identified keratin, and the occurrence of the cross-linking amino acids, desmosine and isodesmosine, in membrane hydrolysates, suggest that elastin or an elastin-like protein is present (Leach and Rucker, 1978; Starcher and King, 1980). The demonstration that the

shell-membrane proteins are resistant to elastase and have an amino acid composition different from that of elastin and other known fibrous proteins (Leach *et al.*, 1981) indicates that the shell membranes of the hen's egg are formed of an as yet uncharacterised fibrous protein. The mantle on the fibres has not been characterised in detail; it probably contains glucose (Baker and Balch, 1962), galactose, mannose and xylulose (Wedral *et al.*, 1974) as well as glucosamine, galactosamine and sialic acid (Cooke and Balch, 1970).

Not only are the shell membranes involved in the diffusion of respiratory gases to and from the chorioallantois (Tullett and Board, 1976; Kayar *et al.*, 1981) and the movement of Ca^{2+} (Coleman and Terepka, 1972) but they play a physical role in protecting the albumen from microbial infection (Bean and MacLaury, 1959; Williams and Whittemore, 1967). Moreover bacteria placed on the inner shell membrane of the air space *in ovo* multiply to a limited extent (Brooks, 1960; Board and Ayres, 1965) unless supplied with Fe^{3+} (Board *et al.*, 1968) or suspended in soil or faecal extracts (Board, 1964). The last mentioned workers noted that the stain following the addition of iron to the shell membranes persisted for several days with incubation at 27 °C. The present study was undertaken with the objectives of identifying the sites(s) in the shell membranes where iron is harboured, and assessing the persistence of iron contamination of the shell membranes.

MATERIALS AND METHODS

Eggs

Good quality eggs and eggs with defective shells and cuticles were obtained from a commercial flock of laying hens, housed in batteries and fed on a proprietary diet. The eggs were used immediately or kept in an incubator (Brinsea Products Ltd, West Brinsea Farm, Congresbury, Avon), maintained at 37·5 °C and 0·60 relative humidity. The eggs were turned automatically every hour.

Glassware

All glassware used in the experiments involving iron was freed of contaminating iron by the following procedure: 1, an overnight soak in 0·1 M HCl; 2, a rinse with deionised, glass-distilled water; 3, a wash with 8-hydroxyquinoline (BDH Ltd) (5 g/l) in chloroform to chelate any iron present (Waring and Werkman, 1942); 4, a rinse in chloroform to remove any traces of chelate; 5, a dry in a hot-air oven to remove chloroform; 6, a double rinse with deionised, glass-distilled water and 7, a dry in a hot-air oven.

Iron solutions

A solution (6 mg/ml) of filter-sterilised (0·45 μm, Oxoid Ltd) $Fe(NH_4)_2(SO_4)_2.6H_2O$ was used to inoculate (0·1 ml) the inner shell membrane of the eggs. The needle of a hypodermic syringe was pushed through the outer shell membrane via a small hole drilled in the shell at the air space. The whole operation was done aseptically and the hole in the shell sealed with hot paraffin wax.

A colloidal iron suspension was prepared according to the method of Rinehart and Abul-Haj (1951). Fifty grams $FeCl_3.6H_2O$ was dissolved in 80 ml glass-distilled water and 40 ml glycerol added slowly. Twenty-two ml ammonia solution (280 ml/l) was carefully and slowly mixed into the iron suspension which was stirred continuously to prevent the formation of excessive precipitate. The whole suspension was dialysed against two changes of distilled water every day for three days—it can be kept for several months at 4 °C. Warm eggs (37·5 °C) placed in this cold iron suspension drew iron through the pores in the shell onto the shell membranes due to the pressure differential generated by the contraction of the egg contents.

Iron determination

Shell membranes containing bound iron were washed six times with deionised water to remove any adhering albumen and solubilised by boiling in ammonium persulphate (20 g/l). Albumen containing iron was mixed with an equal volume of ascorbate (BDH Ltd) (0·2 g/l) in 0·2 M HCl and allowed to stand for 10 min. The protein was precipitated using trichloroacetic acid (110 g/l) and removed by centrifugation (5 000 *g*).

To 0·5 ml of solubilised membrane or egg albumen supernatant was added 0·4 ml ammonium acetate (100 g/l) followed by 0·1 ml of iron reagent containing 3 mg/ml of ferrozine (Sigma Ltd) and neocuproine (Sigman Ltd). The absorbance of the magenta-coloured complex was measured at 562 nm against water. Iron concentrations were calculated from standards containing known amounts of iron processed in the same way as the test samples.

Electron microscopy

Samples of shell membranes for scanning electron microscopy were fixed in gluteraldehyde (20 g/l) in 0·05 M cacodylate buffer, pH 7·0, for 60 min at room temperature followed by dehydration in a series (50 to 100%) of acetone-water mixtures. The samples were then dried by the critical point method using a Polaron 5000 critical drying point apparatus and mounted on aluminium stubs using DAG 915 (Acheson Colloids Co., Prince Rock, Plymouth). The specimens were coated under vacuum with a thin layer of gold/palladium alloy and examined using a Joel 35C (Joel (UK) Ltd, Colindale, London) Scanning electron Microscope C at an accelerating voltage of 25 kV.

Samples of shell membranes for transmission electron microscopy were fixed and stained in gluteraldehyde (20 g/l) containing ruthenium red (1 g/l). After dehydration as described above the membranes were embedded in Taab EM resin (Taab Laboratories Equipment Ltd, Reading, Berkshire) and ultra-thin sections, cut with a glass knife, collected onto uncoated copper grids. Sections were stained with a saturated solution of uranyl acetate in ethanol (700 ml/l) and lead citrate for 15 min each and examined using the Joel 100C Transmission electron Microscope (Joel (UK) Ltd, Colindale, London) at an accelerating voltage of 20 kV. Some sections were stained with the colloidal iron suspension for 4 h before examination with the electron microscope.

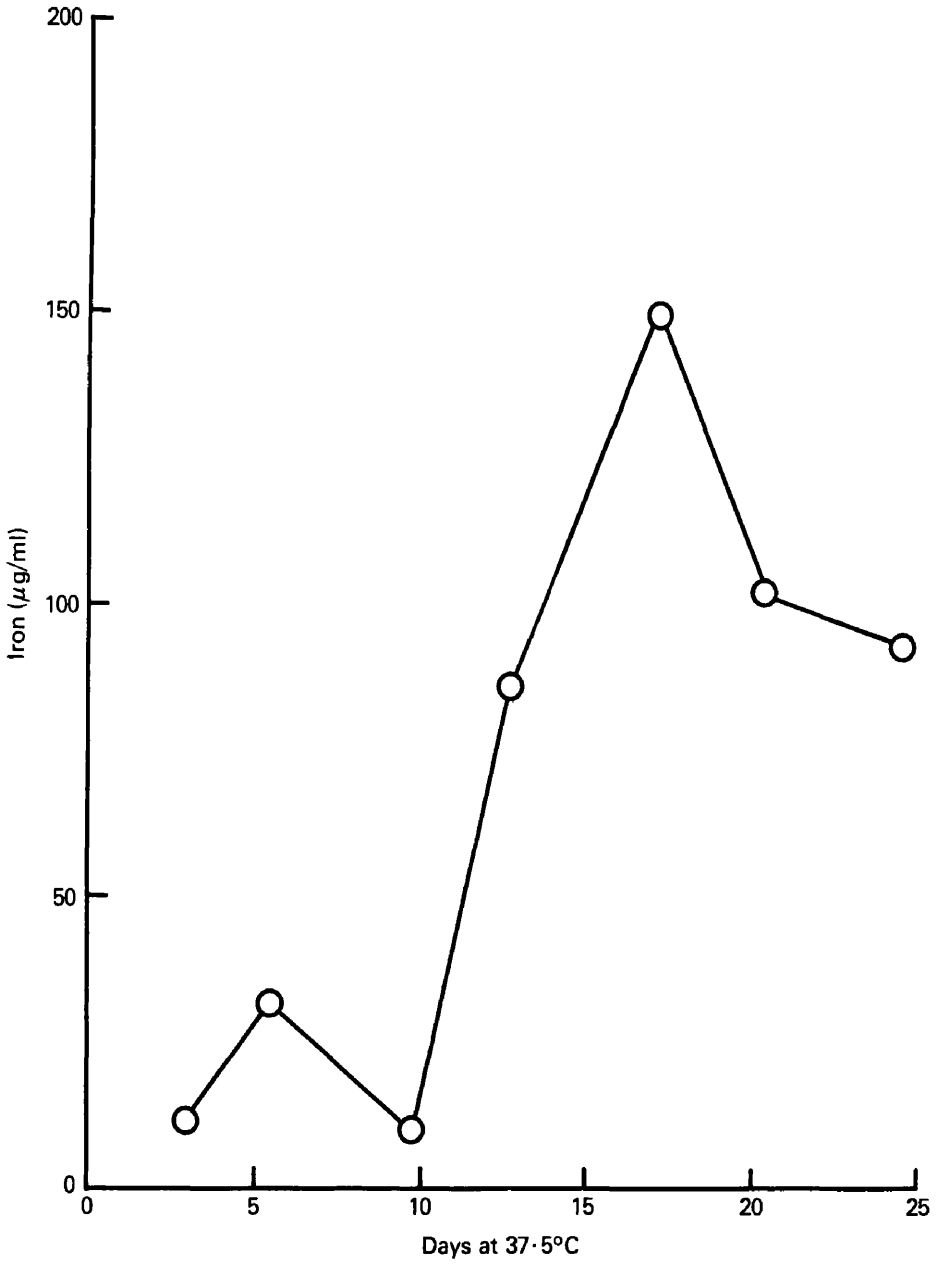

FIG. 2.—Changes in concentration of iron of albumen of incubating infertile eggs following addition of iron to the membranes. Treatment as in Fig. 1, albumen taken, mixed and assayed for iron.

shell membranes. As a consequence, organisms resistant to the antimicrobial agent would have their growth promoted.

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REFERENCES

BAKER, J. R. AND BALCH, D. A. (1962). A study of the organic material of the hen's egg shell. *Biochemical Journal*, **82**: 352–361.

BEAN, K. C. AND MACLAURY, D. W. (1959). The bacterial contamination of hatching eggs and methods for its control. *Poultry Science*, **38**: 693–698.

BELLAIRS, R. AND BOYDE, A. (1969). Scanning electron microscopy of the shell membranes of the hen's egg. *Zeitschrift für Zellforschung und Mikroskopische anatomie*, **96**: 237–249.

BOARD, R. G. (1964). The growth of Gram-negative bacteria in the hen's egg. *Journal of Applied Bacteriology*, **27**: 350–364.

BOARD, R. G. (1980). The avian eggshell—a resistance network. *Journal of Applied Bacteriology*, **48**: 303–313.

BOARD, R. G. AND AYRES, J. C. (1965). The influence of temperature on bacterial infection of the hen's egg. *Applied Microbiology*, **13**: 358–364.

BOARD, R. G. AND HALLS, N. A. (1973). The cuticle: a barrier to liquid and particle penetration of the shell of the hen's egg. *British Poultry Science*, **14**: 69–97.

BOARD, P. A., HENDEN, L. P. AND BOARD, R. G. (1968). The influence of iron on the course of bacterial infection of the hen's egg. *British Poultry Science*, **9**: 211–215.

BROOKS, J. (1960). Mechanisms of the multiplication of *Pseudomonas* in the hen's egg. *Journal of Applied Bacteriology*, **23**: 499–509.

COLEMAN, J. R. AND TEREPKA, A. R. (1972). Fine structural changes associated with the onset of calcium, sodium and water transport by the chick chorioallantoic membrane. *Journal of Membrane Biology*, **7**: 111–127.

COOKE, A. S. AND BALCH, D. A. (1970). The distribution and carbohydrate composition of the organic matrix in hen egg shell. *British Poultry Science*, **11**: 353–365.

GARIBALDI, J. A. AND BAYNE, H. G. (1960). The effect of iron on *Pseudomonas* spoilage of experimentally infected shell eggs. *Poultry Science*, **39**: 1517–1520.

GARIBALDI, J. A. AND BAYNE, H. G. (1962*a*). Iron and the bacterial spoilage of shell eggs. *Journal of Food Science*, **27**: 57–59.

GARIBALDI, J. A. AND BAYNE, H. G. (1962*b*). The effect of iron on the *Pseudomonas* spoilage of farm washed eggs. *Poultry Science*, **41**: 850–853.

HOFFER, A. P. (1971). The ultrastructure and cytochemistry of the shell membrane-secreting region of the Japanese quail oviduct. *American Journal of Anatomy*, **131**: 253–287.

KAYAR, S. R., SNYDER, G. K., BIRCHARD, G. F. AND BLACK, C. P. (1981). Oxygen permeability of the shell membranes of chicken eggs during development. *Respiration Physiology*, **46**: 209–221.

LEACH, R. M. AND RUCKER, R. B. (1978). Studies on the major protein component of egg shell membranes. *Poultry Science*, **57**: 1151.

LEACH, R. M., RUCKER, R. B. AND VAN DYKE, G. P. (1981). Egg shell membrane protein: A nonelastin desmosine/isodesmosine-containing protein. *Archives of Biochemistry and Biophysics*, **207**: 353–359.

MASSHOFF, W. AND STOLPMANN, H. J. (1961). Licht—und elektronenmikroskopische Untersuchungen an der Schalenhaut und Kalkschale des Huhnereies. *Zeitschrift Für Zellforschung und Mikroskopische anatomie*, **55**: 818–832.

MORAN, T. AND HALE, H. P. (1936). Physics of the hen's egg. 1. Membranes in the egg. *Journal of Experimental Biology*, **13**: 35–41.

NARBAITZ, R. AND TELLIER, P. P. (1974). The differentiation of the chick chorionic epithelium: an experimental study. *Journal of Embryology and Experimental Morphogenesis*, **32**: 365–374.

RINEHART, J. F. AND ABUL-HAJ, S. K. (1951). An improved method for histologic demonstration of acid mucopolysaccharides in tissues. *Archive of Pathology*, **52**: 189.

SIMKISS, K. (1968). The structure and formation of the shell and membranes. In: *Egg Quality—A Study of the Hen's Egg*, pp. 3–25, edit. CARTER, T. C. Edinburgh, Oliver and Boyd.

STARCHER, B. C. AND KING, G. S. (1980). The presence of desmosine and isodesmosine in eggshell membrane protein. *Connective Tissue Research*, **8**: 53–55.

TULLETT, S. G. AND BOARD, R. G. (1976). Oxygen flux across the integument of the avian egg during incubation. *British Poultry Science*, **17**: 441–450.

TUNG, M. A. AND RICHARDS, J. F. (1972). Ultrastructure of the hen's egg shell membranes by electron microscopy. *Journal of Food Science*, **37**: 277–281.

WARING, W. S. AND WERKMAN, C. H. (1942). Growth of bacteria in an iron-free medium. *Archives of Biochemistry*, **1**: 303–310.

WEDRAL, E. M., VADEHRA, D. V. AND BAKER, R. C. (1974). Chemical composition of the cuticle, and the inner and outer shell membranes from eggs of *Gallus gallus*. *Comparative Biochemistry and Physiology*, **47B**: 631–540.

WILLIAMS, J. E. AND WHITTEMORE, A. D. (1967). A method for studying microbial penetration through the outer structures of the avian egg. *Avian Diseases*, **11**: 467–490.

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CUTICLE, SHELL POROSITY AND WATER UPTAKE THROUGH HENS' EGGSHELLS

N. H. C. SPARKS AND R. G. BOARD

School of Biological Sciences, University of Bath, Bath, BA2 7AY, England

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Abstract 1. There was no correlation between water uptake by a warm egg (37 °C) immersed in iced water and the shell's porosity as measured by water vapour conductance.

2. Eggs lacking cuticle on the shell took up more water than those having cuticle. There was no correlation between water uptake and the water vapour conductance of cuticle-less eggshells.

3. In general there was no correlation between the amount of water taken up by an egg and the weight of cuticle on its shell. With some eggs there was an association between water uptake and the extent of fissuring of the cuticle.

4. It was postulated that the pore canals impede water flow such that osmotic forces acting across the shell membranes do not influence water uptake by eggs.

INTRODUCTION

It is accepted that a very small proportion, probably less than 1%, of hens' eggs contain microorganisms at oviposition (Brooks and Taylor, 1955). Thus the breaching of the physical defence offered by the egg's integument (the calcitic shell with its outer covering of cuticle, the inner and outer shell membranes and the limiting membrane) is the first step in the process leading to the rotting of table or incubated eggs (Board, 1966), in the genesis of omphalitis (Harry, 1957) and, perhaps even the colonisation of the alimentary canal, especially the caeca, of newly-hatched chicks by salmonellae (Snoeyenbos *et al.*, 1978). Although Zagaevsky and Lutikova (1944) suggest that the integument is readily breached immediately following oviposition, the majority of studies have been concerned with eggs which were challenged with bacteria following removal from nest boxes.

Water, either as vapour or liquid, appears to be essential for microbial penetration of the integument. Thus a high (95% RH) water content in the atmosphere surrounding an egg is a prerequisite for mould or bacterial growth on the cuticle and subsequent microbial penetration of the pores in the calcitic shell (Board *et al.*, 1979). The studies of Haines and Moran (1941) identified some of the factors which contribute to microbial penetration of the shells of eggs immersed in water. They noted

penetration to be low when water and eggs were of the same temperature but high when the eggs were warmer than the water. The latter observation led them to conclude that a negative pressure was created in the cooling egg because the volume of the contents contracted more than that of the shell, the difference being abolished when contaminated water was sucked through the pores. Indeed, their observations have had a major impact on practices in the poultry industry. Thus in the washing of table eggs, emphasis is given to the need to avoid the generation of a negative pressure—the temperature of the wash water must be greater than that of the eggs (Moats, 1978). An opposing strategy is adopted to free incubating eggs of mycoplasma, warm eggs being immersed in chilled solutions of antibiotics (Alls *et al.*, 1963, 1964).

Although factors contributing to microbial penetration have been known for more than 40 years, little headway has been made in attempts to identify the contribution of the various components, especially their physical attributes, of the integument to the egg's defence. Romanoff (1931) appears to have concluded intuitively that the cuticle played a major role; supporting observations were published by Vadehra *et al.* (1970) and Board and Halls (1973). Other workers (Williams *et al.*, 1968) have concluded that the resistance of the inner shell membrane to microbial penetration is greater than that of the outer one. Such conclusions must be cautious because the experiments did not distinguish between the resistance of the inner shell membrane *per se* and the limiting membrane (Tranter *et al.*, 1983). Interpretation of many studies of bacterial penetration of egg shells is difficult (Board and Halls, 1973) because porosity has been defined in many different ways. The classical studies of the diffusion of water vapour across eggshells by Rahn and his collaborators (Ar *et al.*, 1974) have provided a definition and a simple means of determining it.

This paper presents observations on factors affecting water uptake by hens' eggs of known porosity *sensu strictu*. The study was based on the hypothesis that water uptake, as measured by a gain in weight, provides an index of the effectiveness of an egg's integument in preventing penetration by microorganisms.

MATERIALS AND METHODS

Eggs were obtained from a commercial flock (Ross Ranger) housed in batteries, and were used on the day of collection.

Water vapour conductance

Water loss from the egg was expressed according to the equation (Ar *et al.*, 1974):

$$G_{H_2O} = \frac{M_{H_2O}}{\Delta P_{H_2O}}$$

where G_{H_2O} = Water vapour conductance expressed as mg of water lost per mm Hg of difference in water vapour pressure across the shell per d.
M_{H_2O} = Water loss from the egg (mg/d).
ΔP_{H_2O} = Difference in water vapour pressure across the eggshell (mm Hg).

G_{H_2O} is a measure of the number and size of the pores and is constant once shell formation has been completed.

G_{H_2O} was determined by packing preweighed eggs in desiccant (Drierite 10/20 mesh; Koch Light Laboratories Ltd) contained in a vented desiccator at 37 °C. The eggs were repacked in fresh desiccant daily and reweighed on day 3. As the water vapour pressure around the eggs was 0 mm Hg and the egg contents were assumed to be at the saturated vapour pressure for the relevant temperature, first ΔP_{H_2O} and then G_{H_2O} could be determined.

Water uptake

A modified technique based on principles described by Haines and Moran (1941) and Board and Board (1967) was used. Eggs at 37 °C were weighed and immersed for 15 min in chilled (1 °C) water or a solution (10 g/l) of Edicol supra pea green H (10 g/l) (I.C.I.). The eggs were removed after 15 min and weighed immediately after the shells had been wiped dry.

Cuticle staining, removal and estimation

The cuticle was stained with Edicol supra pea green H (Board and Halls, 1973). The eggs were immersed for 15 min in a 0·34 M alkaline (pH 7·5) solution of ethylenediaminetetraacetic acid (EDTA). The cuticle was removed with a fine jet of water. Cuticle was estimated by removing it with EDTA, harvesting it on a glass microfibre filter, washing it and drying to constant weight.

Electron microscopy

Samples of shell were placed in liquid nitrogen and freeze dried. They were mounted on aluminium planchettes and sputter coated *in vacuo* with gold/palladium alloy. The samples were examined with a Jeol 35C scanning electron microscope (S.E.M.). Some samples of shell fixed in 0·5 M gluteraldehyde in 0·2 M cacodylate buffer (pH 7·0) for 60 min at room temperature were dehydrated in a series (50 to 100%) of acetone solutions. The samples were dried using a critical point dryer (Polaron Equipment Ltd), mounted on aluminium planchettes and coated *in vacuo* with a gold/palladium alloy.

Cuticle crack length

Samples of shell were freeze dried and micrographs of the cuticle taken using a Jeol 35C S.E.M. at a magnification of times 130. A transparent film with a ruled grid (9 × 6 squares) was laid over the micrograph and the cuticle cracks traced for a randomly-determined square on to tracing paper. Crack length was measured with a map measure (Polco Products Ltd).

Pore counts, cone counts and distribution

Acid etching (Tyler, 1969) was used to determine pore numbers and their distribution was assessed by the method of Tyler and Fowler (1978). This involves the

measurement of nearest neighbours (r_A) in a known area of shell (A). From the mean ($\bar{r}_A$), a value Q was determined:

$$Q = \frac{A}{\pi(\bar{r}_A^2)}$$

When Q is plotted against N (number of cones or pores), a random distribution would give a line bisecting the origins of the axes with a slope of 1·00. Points above this line are evidence of aggregation and those below of uniformity.

Cone counts were made by the method of Tyler and Fowler (1978), using scanning electron micrographs instead of drawings made with a projection microscope.

Shell-less eggs

Shell-less eggs were immersed in water. At regular intervals they were removed, wiped dry and weighed.

Shell thickness

An anvil-jawed micrometer (hemispherical jaws) was used to measure shell thickness of shells from which the membranes had been removed, the mean of 3 readings obtained from separate shoulder pieces being taken.

RESULTS

Although measurement of water vapour conductance by the method of Ar *et al.* (1974) can be used to determine eggshell porosity, the actual location of the pores needs to be stressed. The outer orifices are covered and often roughly plugged with cuticle—a fissured layer formed from spheres of glycoproteins (Wedral *et al.*, 1974). The inner orifices are between the cones, the tips of which fuse with the outer shell membranes, which are elastic and semipermeable (Fig. 1).

When untreated eggs at 37 °C were immersed in ice cold water for 15 min, the amount of water taken up varied from 3 to 415 mg/egg (number of eggs, $n = 90$). There was no correlation between the amount of water taken up and shell porosity (Fig. 2). Nor was there any correlation when the number of pores per shell (8 to 20 $\times$ 10^3) was regressed against water uptake ($r = 0{\cdot}03$; $P < 0{\cdot}05$; $n = 70$). That pores *per se* had little influence on water uptake by warm eggs in chilled water was suggested by the separation and numbers of pores and cones (Table). We concluded that eggshells having relatively few, large but widely-distributed pores were not especially vulnerable to flooding with water and *vice versa*. In practice this is true only when all pores, 10 000 or more per egg, are considered. Subsequent studies with chilled dye solutions showed that large patches of shell membrane were stained in some eggs; it was inferred that these occurred at the internal orifice of a pore with a large canal, formed as a consequence of a localised malformation in the cone layer, and referred to as "patent pores" (Board, 1980). Large patches of stained membrane were also found underlying a part of the shell with a projecting calcareous lump. Such lumps, sometimes persisent

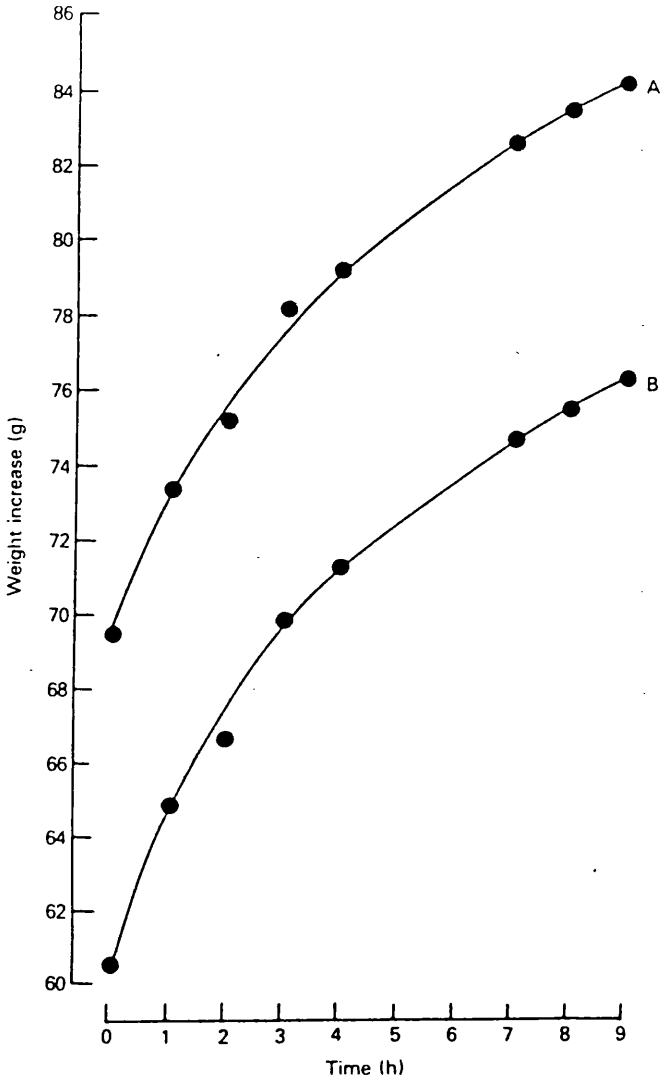

FIG. 1.—Water uptake by a shell-less egg. The egg was immersed in water and the weight increase measured (A). The egg was then removed and allowed to stand overnight before being immersed a second time (B).

traits of the eggs of particular hens, cause malformation of the pores in the adjoining shell.

The lack of evidence associating porosity with water uptake led us to examine the cuticle, using ice cold solutions of Edicol pea green. Figure 2 shows that warm eggs freed of cuticle by EDTA took up more water than untreated controls. As the G_{H_2O} of the former did not differ significantly from the latter ($P > 0{\cdot}05$), the chelating agent could not have appreciably modified the pore canals. The use of the stain led to the identification of certain factors that negate the water resistance conferred on shells by the cuticle. Thus one or two malformed pores in a shell clothed with cuticle caused appreciable water uptake. Some of the eggs which gained weight (up to 415 mg)

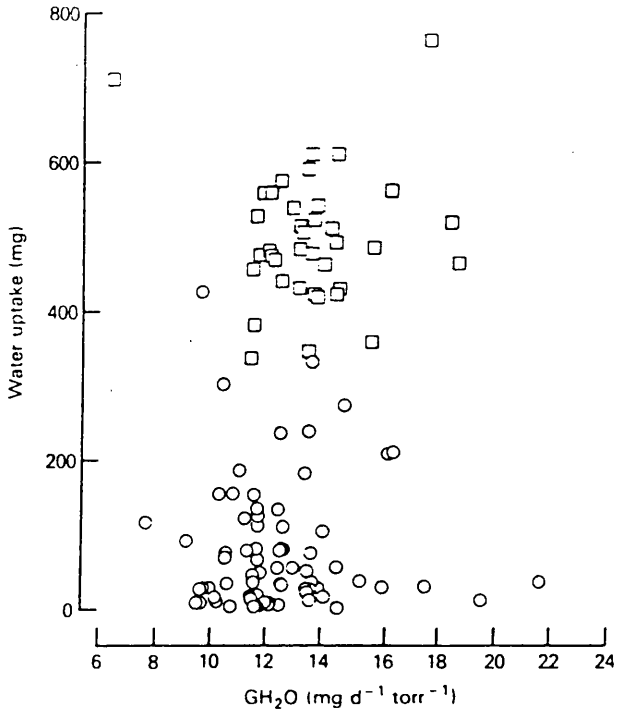

FIG. 2.—Removal of cuticle (□) does not change the G_{H_2O} values compared with untreated (○) eggs although water uptake is increased.

following immersion did not have an intact cuticle, indeed naturally cuticle-less eggs behaved in the same way as those from which cuticle had been removed with EDTA. A poor cuticle was characteristic of the eggs of some hens, such as No. 8, at the end of their laying cycle (Fig. 3). Others had damaged cuticles, commonly a scrape probably caused by damage to the moist cuticle of oviposition as the egg rolled down the inclined cage floor. Such eggs comprised about 40% of those in this study. The use of the stain showed also that large amounts of water were taken up by eggs with hair-line cracks.

TABLE

The relationship between pore numbers, cone numbers and their distribution in hens' eggshells[1]

Relationship of distribution factor[2] to:	Correlation coefficient	t test	Number of eggs examined
Number of pores	0·90	$P < 0{\cdot}001$	46
	0·96	$P < 0{\cdot}001$	9
Number of cones	0·95	$P < 0{\cdot}001$	11
	0·93	$P < 0{\cdot}001$	29

[1] The analytical methods of Tyler and Fowler (1978) were used.
[2] Distribution factor Q was determined from:

$$\frac{A}{\pi \cdot \bar{r}_4^2}$$

Where A = surface area; $\bar{r}_4$ = mean of nearest neighbour measurements of cones or pores. A correlation of 0·90 accounts for 81% of the variation ($0{\cdot}90^2 \times 100$).

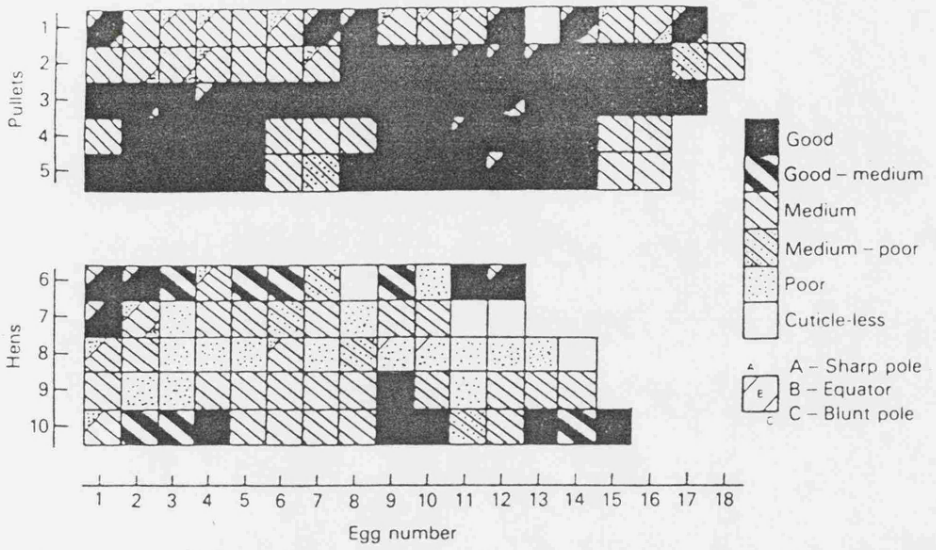

FIG. 3.—Diagrammatic representation of the cuticle quality as determined by staining with Edicol Supra Pea Green H.

No correlation was evident when the amount of water taken up by an egg was regressed against the amount of cuticle per cm^2 of shell ($r = 0{\cdot}50$, $P < 0{\cdot}05$; $n = 48$).

The extent of fissuring of the cuticle might be a possible cause of the lack of an association between the water uptake of an egg and its cuticle quality as determined gravimetrically. This surmise was tested by determining the water uptake of 50 eggs and selecting 5 with high, intermediate or low water uptake. The length of the fissures in the cuticle of each egg was measured and regressed against water uptake. A significant correlation was noted ($r = 0{\cdot}9$; $P < 0{\cdot}05$). No such correlation was evident when the fissure lengths in the cuticles of 24 eggs were regressed against the amount of water each had taken up. Through determining the contact angle (θ), the dimensions of the fissures as well as the microstructure of their radial faces would obviously influence the effectiveness of the cuticle as a barrier to water. It is noteworthy,

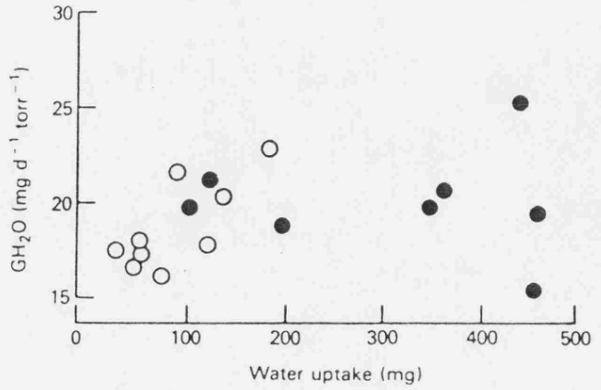

FIG. 4.—Lowering the water's surface tension with a surfactant, Triton X-100 (●) leads to an increase in water uptake compared with the controls (○).

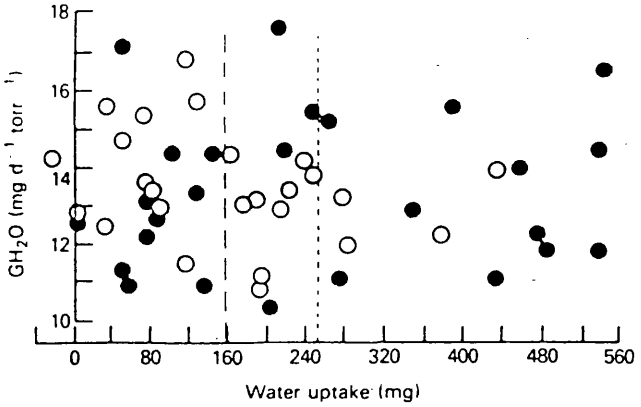

FIG. 5.—Degassing the water (●) has a similar effect, controls (○); —— control mean; --- degassed mean.

therefore, that the effectiveness of the cuticle was diminished when a surfactant was added to water (Fig. 4). Figure 5 shows that water uptake was also increased by degassing, suggesting that gas may normally come out of solution at the water:cuticle interface, thereby satisfying in part the pressure differential generated by a warm egg contracting in cold water.

Cuticle, although the major impediment to water penetration, is not the sole contributor. If it were, a clear-cut relationship between water uptake and G_{H_2O} of shells freed of cuticle by EDTA would be expected (Fig. 2). These results indicate that some feature of the pore canals plays a role, albeit minor, in impeding water movement; some of our observations suggest that resistance to water flow, a drag factor, may be involved. The semi-permeable and elastic properties of the shell membranes of naturally shell-less eggs are evident from Fig. 1. Thus there would appear to be the potential for the column of water in a flooded pore canal to be drawn in by osmotic forces acting across the shell membranes. The large areas of stained membranes, together with appreciable water uptake by eggs whose shells containe malformed pores or hair-line cracks, is taken to be evidence of osmotic forces acting across the shell membrane. The tendency of a surfactant (Fig. 4) to enhance water uptake by eggs may also be evidence of the drag factor postulated above.

DISCUSSION

The biological consequence of G_{H_2O} is related to two conflicting requirements, a porosity that allows sufficient gaseous diffusion to support embryogenesis but does not cause excessive water loss and dehydration. The embryos in very porous shells can be protected from excessive water loss through the control of humidity in the incubator; those in shells at the other extreme will be asphyxiated. Studies of the hatching eggs of commercial flocks of turkeys and hens have shown (Tullett, 1981, Tullett and Burton, 1982), however, that the majority of eggshells have a porosity within a range that accommodates this conflict. Although porosity has a central role in eggs being incubated, this study is the first to demonstrate that it has a negligible influence on the amount of water taken up when a warm egg contracts in iced water. The results presented here supported the conclusion (Board and Halls, 1973) that the cuticle confers water resistance to the hen's egg. Moreover, as the cuticle does not influence G_{H_2O} apprecia-

bly, a breeding programme concerned with the latter need not involve the former.

The biological functions of the cuticle have attracted little attention. Lack (1968), discussing avian eggs generally, thought that material on the outer surface of the calcitic shell was an adaptation that fitted eggs laid in wet places to an inimical environment, but did not specify the dangers to which the embryos would be exposed should the shell lack such adaptations. In a discussion of Lack's (1968) hypothesis, Board (1982) suggested that, from the viewpoint of gaseous diffusion, the integument on avian eggs can be considered as a series of diffusion pathways or resistances, and that modifications of the outer surface of the shell are adaptations that ensure optimal gaseous diffusion in nests containing debris which might occlude the pore canals. Such a role for the cuticle was evident in a study of guinea fowl eggs incubated in the wild (Board and Perrott, 1982). The present communication is, we believe, the first to demonstrate conclusively that the cuticle on the hen's eggshell does not influence G_{H_2O} but does protect the underlying pore canals from flooding and, by implication, bacterial contamination. These functions have important practical implications also. The water resistance poses a problem for those who wish to rid incubating eggs of mycoplasma by flooding at least some of the pores with antibiotics (Alls *et al.*, 1964). In addition, attempts to prevent egg transmission of *Salmonella* spp. may be negated by imperfectly-formed or damaged cuticle allowing the organisms to penetrate the shell.

There is little evidence concerning the synthesis of cuticle in domestic hens and only one study (Ball *et al.*, 1975) which suggests that cuticle "quality" may be heritable. Only one method, contraction of a warm egg in iced water, was used in attempts to overcome the cuticle's resistance to water, because in the poultry industry it is the principal cause of bacterial infection of eggs, or at least of those infected after collection from the nest (Tranter and Board, 1982). We have examined the role of the cuticle by comparing the water uptake of normal eggs with those cuticle-less at oviposition or rendered cuticle-less by EDTA. The significant differences suggest that a simple staining reaction could be used in a breeding programme designed to reduce the incidence of naturally cuticle-less eggs. Results from a survey of eggs laid by individually-caged birds suggest that it would be useful to follow gross changes in cuticle deposition during a laying cycle. Our observations of extensive water uptake by some eggs with large fissures suggest that a method of assessing cuticle quality other than its staining potential would be needed to select for those that were particularly water resistant. In addition, malformation of the shell can undermine the function of the cuticle; patent pores and penetrating calcareous lumps were two such malformations identified. The latter malformation appeared to be a trait of particular hens and, if heritable, could be selected against. The cause of patent pores will remain uncertain until all the factors contributing to core formation, initiation and growth have been identified; even then the complexities of shell initiation may be such that some large pores will form in an otherwise normal egg.

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REFERENCES

Ayres, A. A., Benton, N. J., Kraus, W. C. and Cover, M. S. (1963). The mechanics of treating hatching eggs for disease prevention. *Avian Diseases*, **7**: 89–97.

Ayres, A. A., Cover, M. S., Benton, W. J. and Kraus, W. C. (1964). Treatment of hatching eggs for disease prevention—factors affecting permeability and a visual detection of drug absorption. *Avian Diseases*, **8**: 245–256.

Ar, A., Paganelli, C. V., Reeves, R. B., Greene, D. G. and Rahn, H. (1974). The avian egg: water vapour conductance, shell thickness and functional pore area. *Condor*, **76**: 153–158.

Ball, R. F., Logan, V. and Hill, J. F. (1975). Factors affecting the cuticle of the egg as measured by intensity of staining. *Poultry Science*, **54**: 1479–1484.

Board, P. A. and Board, R. G. (1967). A method of studying bacterial penetration of the shell of the hen's egg. *Laboratory Practice*, **16**(29): 471–472, 482.

Board, R. G. (1966). Review article; the course of microbial infection of the hen's egg. *Journal of Applied Bacteriology*, **29**: 319–341.

Board, R. G. (1980). The avian eggshell—a resistance network. *Journal of Applied Bacteriology*, **48**: 303–313.

Board, R. G. (1982). Properties of avian eggshells and their adaptive value. *Biological Reviews*, **57**: 1–28.

Board, R. G. and Halls, N. A. (1973). The cuticle: a barrier to liquid and particle penetration of the shell of the hen's egg. *British Poultry Science*, **16**: 69–91.

Board, R. G. and Perrott, H. R. (1982). The fine structure of the outer surface of the incubated eggshells of the Helmeted guinea fowl (*Numida meleagris*). *Journal of Zoology, Lond*, **196**: 445–451.

Board, R. G., Loseby, S. and Miles, V. R. (1979). A note on the microbial growth on hen eggshells. *British Poultry Science*, **20**: 413–420.

Brooks, J. and Taylor, D. I. (1955). Eggs and egg products. *Report of the Food Investigation Board*, No. 60. London, H.M.S.O.

Haines, R. B. and Moran, T. (1941). Porosity of, and bacterial invasion through the shell of hen's egg. *Journal of Hygiene*, **37**: 453–461.

Harry, E. G. (1957). The effect on embryonic and chick mortality of yolk contamination with bacteria from the hen. *Veterinary Record*, **69**: 1433–1441.

Lack, D. (1968). Ecological adaptations for breeding in birds. London, Methuen.

Moats, W. A. (1978). Egg washing—a review. *Journal of Food Protection*, **41**: 919–925.

Romanoff, A. L. (1931). Why some eggs do not hatch. *Cornell University Bulletin No. 205*.

Snoeyenbos, G. H., Weinack, O. M. and Smyser, C. F. (1978). Protecting chicks and poults from salmonellae by oral administration of "normal" gut microflora. *Avian Diseases*, **22**: 273–287.

Tranter, H. S. and Board, R. G. (1982). The antimicrobial defence of avian eggs: biological perspective and chemical basis. *Journal of Applied Bacteriology*, **4**: 295–338.

Tranter, H. S., Sparks, N. H. C. and Board, R. G. (1983). Changes in structure of the limiting membrane and in oxygen permeability of the chicken egg integument during incubation. *British Poultry Science*, **24**: 537–547.

Tullett, S. G. (1981). Theoretical and practical aspects of eggshell porosity. *Turkeys*, **29**(4): 24–28.

Tullett, S. G. and Burton, F. G. (1982). Factors affecting the weight and water status of the chick at hatch. *British Poultry Science*, **23**: 361–269.

Tyler, C. (1969). The distribution of pores in the eggshells of the domestic fowl: a further study. *British Poultry Science*, **10**: 375–380.

Tyler, C. and Fowler, S. (1978). The distribution of organic cores, cones, cone junctions and pores in the eggshells of wild birds. *Journal of Zoology, Lond*, **186**: 1–14.

Vadehra, D. V., Baker, R. C. and Naylor, H. B. (1970). Role of cuticle in spoilage of chicken eggs. *Journal of Food Science*, **35**: 5–6.

Wedral, E. M., Vadehra, D. V. and Baker, R. C. (1974). Chemical composition of the cuticle, and the inner and outer shell membranes from eggs of *Gallus gallus*. *Comparative Biochemistry and Physiology*, **47B**: 631–640.

Williams, J. E., Dillard, L. H. and Hall, G. O. (1968). The penetration patterns of *Salmonella typhimurium* through the outer structure of chicken eggs. *Avian Diseases*, **12**: 445–466.

Zagalsky, J. S. and Lutikova, P. O. (1944). Sanitary measures in the egg breaking plant. *U.S. Egg Poultry Magazine*, **50**: 17–20, 43–46, 75–77, 88–90, 121–123.

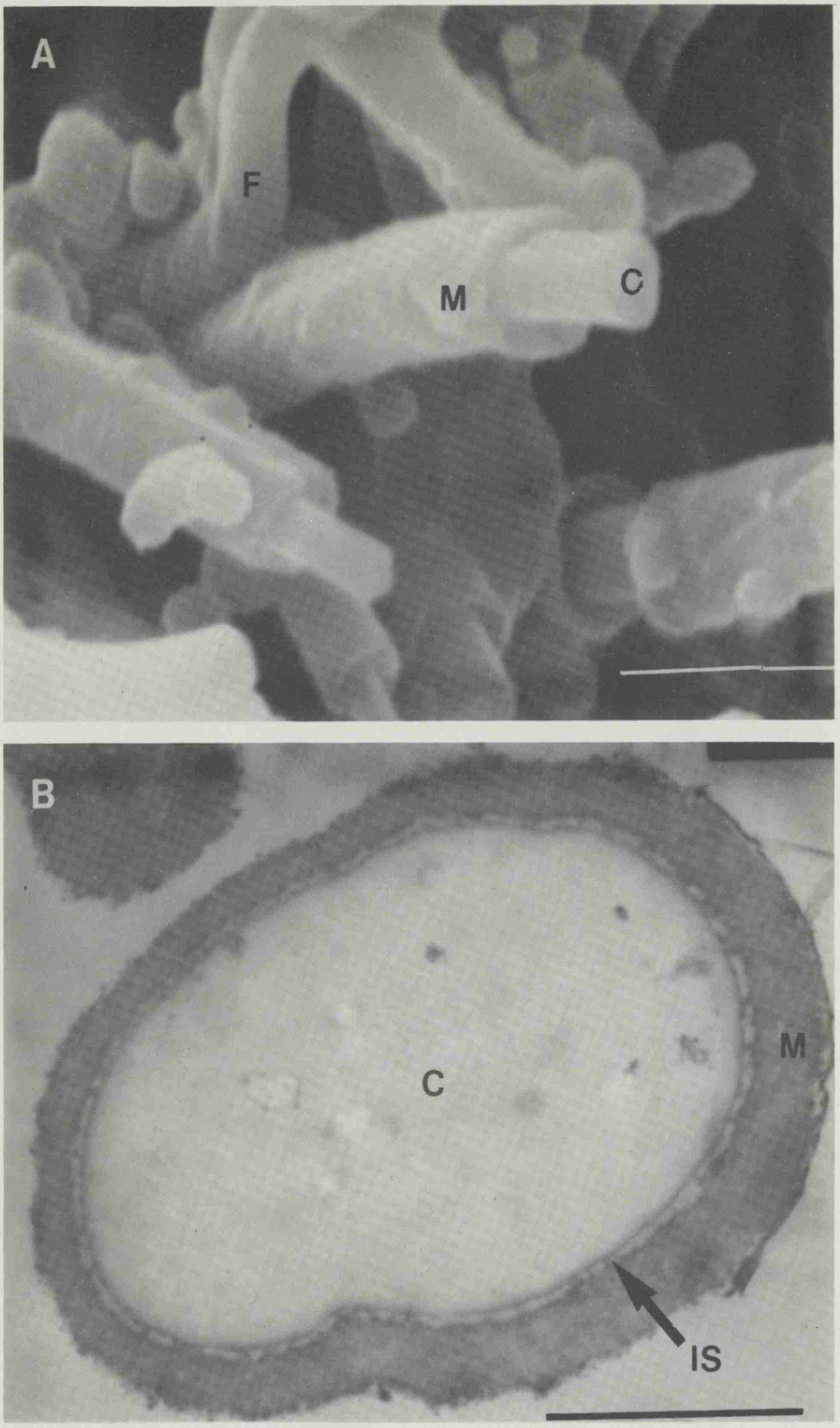
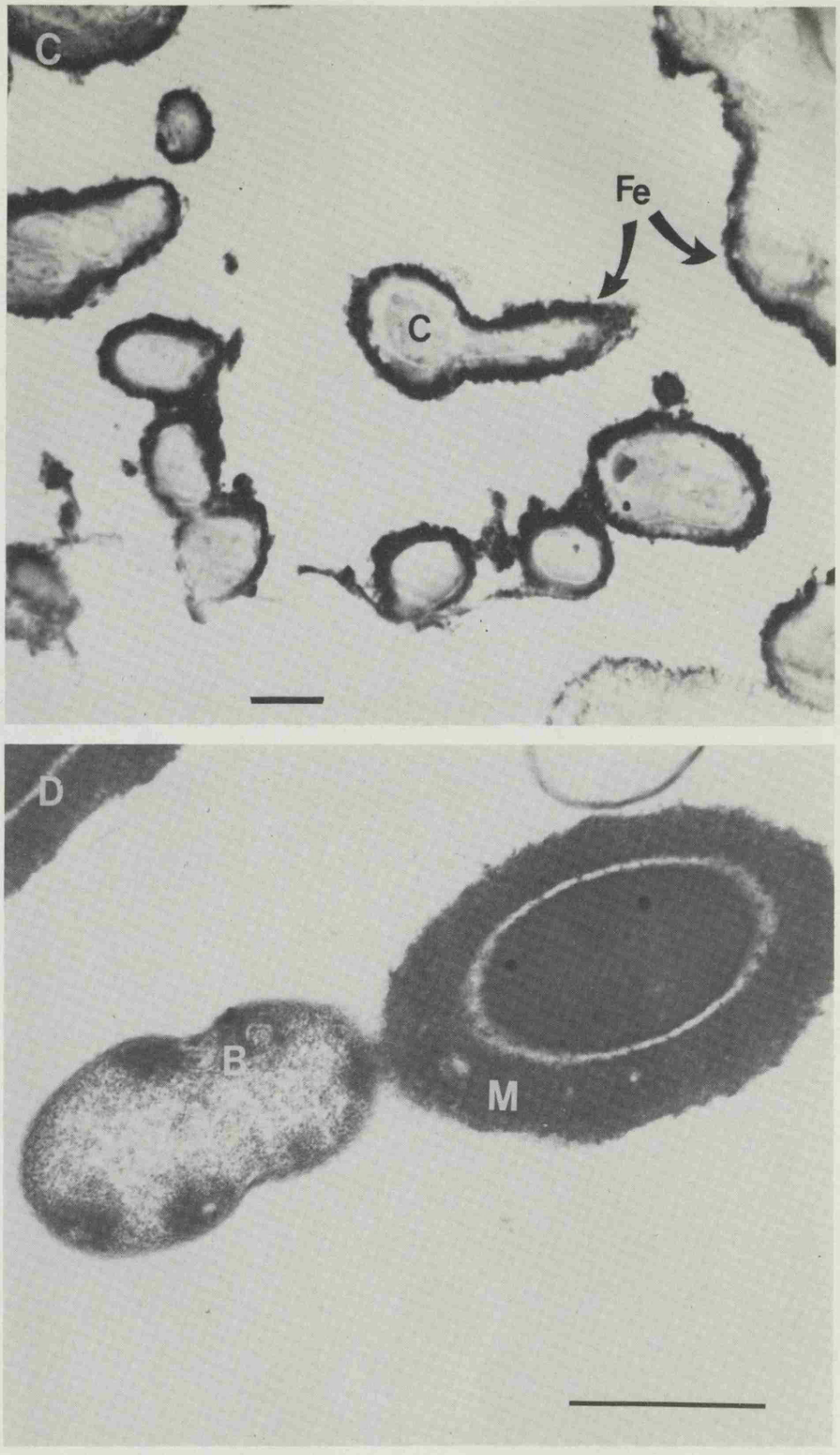


PLATE-FIG. 1.—A. The fibres (F), medulla (M) and core (C) of the shell membranes of the hen's egg shell as seen in scanning electron microscopy; bar marker = 1·00 μm. B. A transverse section of a fibre of the shell membranes of the hen's egg examined with transmission electron microscopy: C, core; M, medulla and IS intrafibrial space; bar marker = 1·00 μm. C. A transverse section of shell membranes of hen's egg treated with colloidal iron and examined with transmission electron microscopy: C, core and Fe, iron-impregnated medulla; bar marker = 1·00 μm. D. A transverse section of shell membranes of hen's egg showing a bacterium (B) adhering to the mantle of the shell membrane (M); bar marker = 1·00 μm. (PLATE-FIG. 1.—continued)



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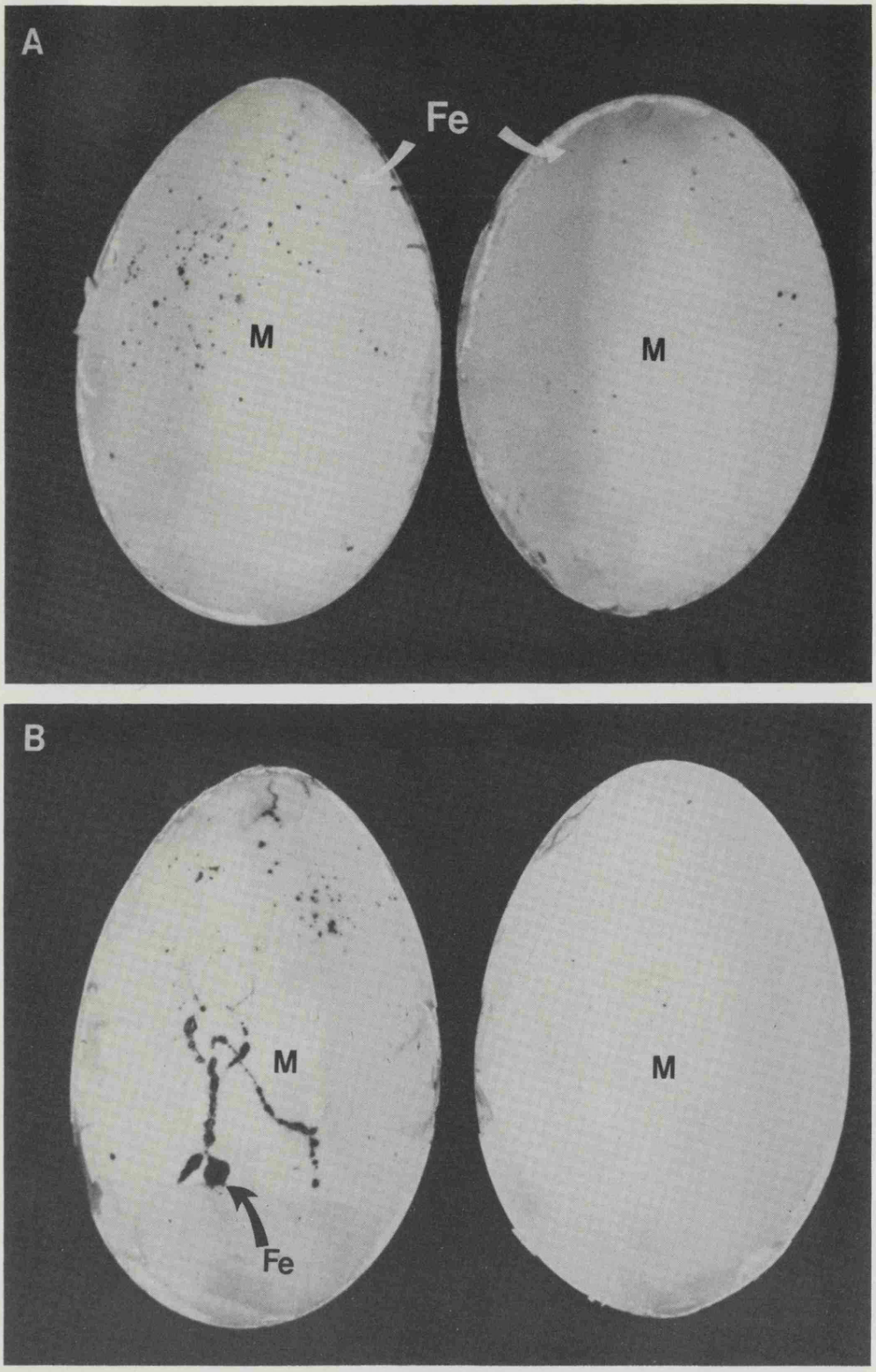
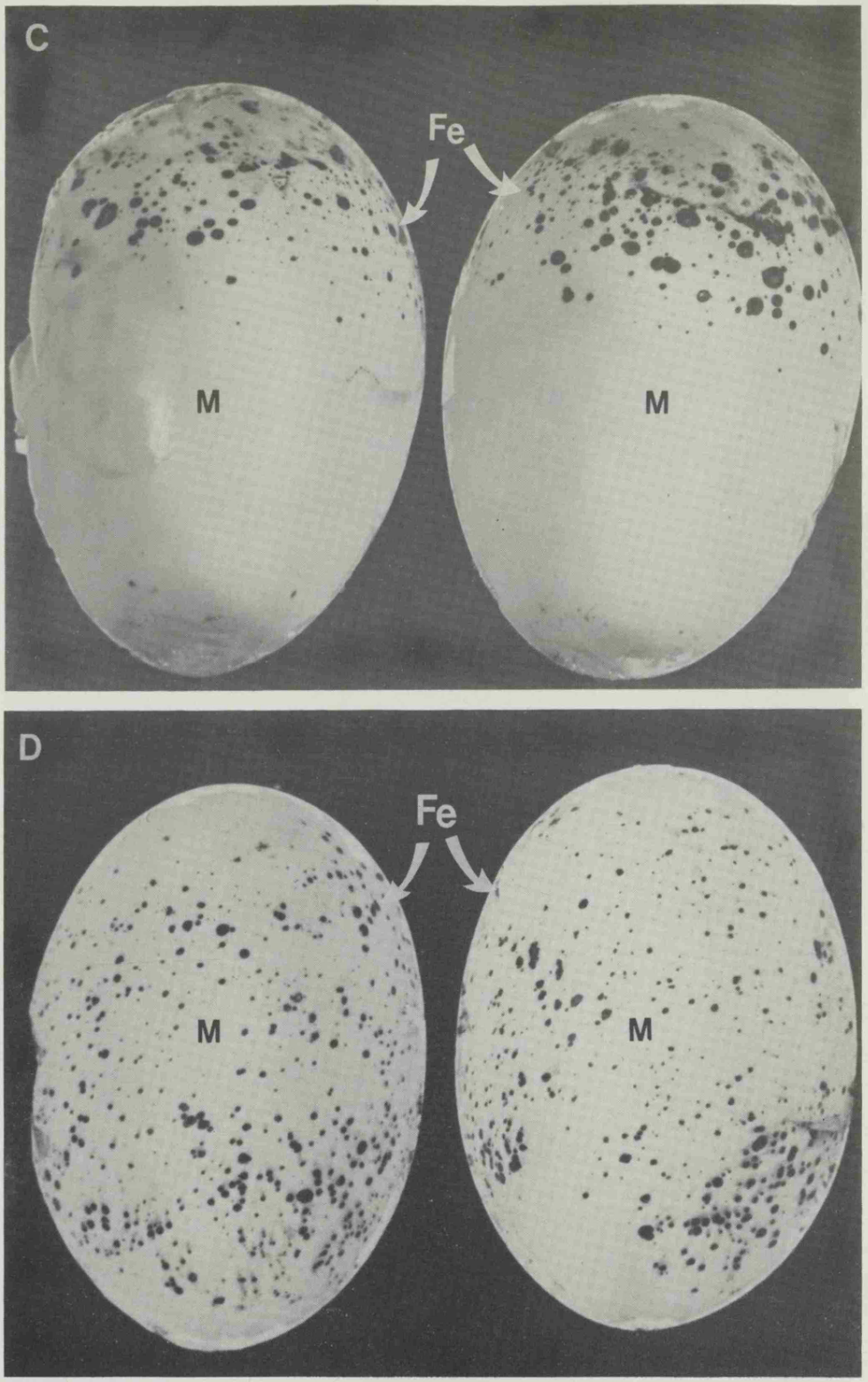


PLATE-FIG. 2.—Hens eggs at 37 °C were immersed for 10 min in ice-cold colloidal iron, the shells cut in half longitudinally, the membranes (M) washed with deionised water and flooded, for 5 min, with potassium ferrocyanide (10 g/l) in 0·1 M HCl, a Prussian blue stain developed (Fe) where iron had penetrated the shell and contaminated the underlying shell membranes. A. An egg with well-formed shell and cuticle. B. An egg of which the shell had been deliberately cracked. C and D. Misshapen eggs with poor cuticles.

(PLATE-FIG. 2.—continued)



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